(2)

AD----------

METABOLISM, MASS SPECTRAL ANALYSIS AND MODE OF ACTION OF TRICHOTHECENE MYCOTOXINS

ANNUAL REPORT

12 OCTOBER 1988

CHESTER J. MIROCHA
ROBERT J. PAWLOSKY
ROLAND GUNTHER

SUPPORTED BY

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

FORT DETRICK, FREDERICK, MARYLAND 21701-5012

CONTRACT NO. DAMD17-85-C-5204

UNIVERSITY OF MINNESOTA
ST. PAUL, MN 55108

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

88 12 14 034

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Minnesota; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): St. Paul, Minnesota 55108	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-85-C-5204
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 63763A; PROJECT NO. 3M2-63763D807; TASK NO. AH; WORK UNIT ACCESSION NO. 046

11. TITLE (Include Security Classification)

(U) Metabolism, Mass Spectral Analysis and Mode of Action of Trichothecene Mycotoxins

12. PERSONAL AUTHOR(S)

Chester J. Mirocha, Robert J. Pawlosky, and Roland Gunther

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 7/15/87 TO 7/14/88	1988 October 12	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA 1, Mycotoxins, T-2 toxin, Mass spectra, metabolism
06	11		
06	04		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The theoretical aspects of hybrid, tandem mass spectrometry is presented in the introduction with its application to multiple reaction monitoring. In summary, the desired parent mass fragment is transmitted through the sectoring portion of the instrument. When it reaches the collision chamber, it is fragmented into daughter ions in the environment of argon gas and voltage. The daughter ion fragments are unique for the compound in question; they are detected by the second quadrupole.

Using the principles of tandem mass spectrometry described above, a method of analysis for T-2 toxin (trifluoroacetate derivative) in blood was developed. The parent fragments used was m/z+ 478 and the daughters that were monitored were m/z+ 180, 138 and 121. Deuterated internal standards were used for quantitative purposes. The TFA derivative of T-2 toxin was detected in whole blood at concentrations of 1, 10 and 20 parts per billion. This same method was used for quantitation of blood samples amended with T-2 toxin at concentrations of 1, 3 and 5 parts per billion. A linear plot of the quantitation is shown.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301-663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

19. ABSTRACT (Continued)

Similar procedures were used for the detection of HT-2 toxin (trifluoroacetate derivative) in whole blood. The parent fragments ion used was m/z+ 532 and the daughters were 180, 138 and 121. The deuterated standard had a mass of 535 and yielded daughters of 121, 138 and 183. A linear relationship was shown for analysis of whole amended with HT-2 toxin at 1, 10 and 20 parts per billion.

Methods used for the synthesis of deuterated T-2, HT-2 and acetyl-T-2 toxins were shown. Their mass spectra and analysis from a biological matrix are presented.

Wortmannin is a natural toxic product produced by Fusarium oxysporum and F. sambucinum. The pathology of these toxins is presented. Pathological lesions in the heart, stomach, thymus and bladder were described.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, Revised 1985).

TABLE OF CONTENTS

ANALYSIS OF T-2 TOXIN IN A BIOLOGICAL MATRIX USING MULTIPLE REACTION MONITORING

The traditional analysis of a biological mixture by mass spectrometry involves the union of a gas liquid chromatograph with a mass spectrometer and analysis of the resolved effluent by either full scan or the recording of selected ions. The latter methodology is sensitive and selective but suffers from the interference presented by the biological matrix. With the advent of tandem mass spectrometry, greater flexibility in the elimination of the effects of a biological matrix is possible. The example used here is that of the trifluoroacetate derivative of T-2 toxin. Thus, a single or multiple selection of parent ions ($m/^{3+}$ 478) is made and allowed to pass through the first analyzer (sectoring portion) of the tandem mass spectrometer into the second mass spectrometer, in this case a quadrupole. Here the parent fragment undergoes collision activated decomposition, under the influence of argon gas and voltage (collision energy) into daughter ions which are detected by the thrid mass spectrometer (quadrupole). The daughters generated from the parent $m/z^{+}478$ of T-2-TFA are 180, 138 and 121. They are unique and give definitive proof for the presence of T-2 toxin. There is the possibility that other 478 fragments may be present in the mixture that have the same retention time as T-2-TFA derivative. In this case, the daughters generated will be different from that of T-2. The method is called multiple reaction monitoring. It is highly accurate and can detect T-2-TFA in blood or urine at a concentration of 1 ppb.

The analytical history of the trichothecene mycotoxins consists

of a list of various physical and chemical methods each one of which was aimed at improving sensitivity, elimination of interfering substances and ease of execution (Mirocha et al. 1986a). The methods used included thin-layer and gas-liquid chromatography, high pressure liquid chromatography, and enzyme linked immunoassay. Except for the latter, the major problem encountered was sensitivity at low concentrations (parts per billion) because of the interference of substances in biological matrix. Thus analysis of blood presented a problem different from that found in urine or in corn or formulated feed samples. Moreover, in the analysis of blood from a pig versus that of a sheep or cow, one encountered different interfering substances and complications so that a method satisfactory for one was not necessarily adequate for the other. Analysis of a biological mixture affects both quantitative as well as qualitative results (retention time and mass spectrum) because of the biological matrix. The goal in analysis is to eliminate the matrix effect in order to achieve precise qualitative and quantitative results.

The use of combination gas chromatography/mass spectrometry assisted the analytical chemist by eliminating the guess work in using retention time as a criterion of identification (Vesonder and Ro wedder, 1986). The use of the full scan mass spectrum (Mirocha et al. 1986b) as a criterion gave definitive information which was further implemented by use of Selective Ion Recording (SIR). It was recommended that in SIR a minimum of three ions should be used in detection but in practice often one or two were used leading to erroneous identification. SIR improved sensitivity so that part per

billion concentrations of pure sample could be detected unequivocally but it suffered from interference of peaks having the same retention time as the component of a mixture being detected. Moreover, quantification suffered because of erratic numbers generated by the effect of the biological matrix.

Combination gas chromatography/mass spectrometry is still the method of choice in analysis but recent advancement of technology has given us a new analytical dimension with the use of tandem mass spectrometry. The latter instrument now gives us the advantage of eliminating excessive matrix effects by analyzing certain parent ions of the compound to be analyzed and studying its daughters or secondary ions (Plattner 1986). The latter are generated by collision of the parent ion with a neutral gas (argon), using a voltage ranging from 1 to 500, and then detecting these products (daughters) by a quadrupole mass spectrometer found downfield from the collision chamber. Thus tandem mass spectrometry has given us another method of analysis called Multiple Reaction Monitoring (MRM) which is the subject of this paper. It will be compared with Selected Ion Recording, Selected Ion Detection or Selected Ion Monitoring (all three terms are synonomous) in order to show advantages of MRM and to show the differences between MRM and SIR.

SIR is traditionally used in conjunction with resolution of a mixture on a capillary column and then selectively detecting certain mass fragments of the molecule in question. By way of example, in the analysis of the trifluoroacetate derivative of T-2 toxin, electron impact values of the molecular ion 562, and the m/z+ 401 and 180 are

selected. This means that when T-2-TFA is detected, the three selected fragment values should be coincident with the retention time of T-2-TFA and the ratio of ion intensities should be correct. Often, interfering substances will have the same retention time and throw off the ratios making identification difficult. On the other hand, MRM is based on the selectivity obtained from adjusting the accelerating voltage of the first mass spectrometer (MS1) to allow passage of one parent ion into the collision chamber in quadrupole number one (MS2). The selected parent collides with the argon (mono atomic molecules) where collision activated decomposition occurs. The daughter ions pass on to quadrupole number 2 where their mass analysis occurs. (Consult Figure 1 for a schematic of the tandem mass spectrometer and the different analyzer and reaction regions). Using T-2-TFA as an example, the parent fragment (m/z+478) will yield daughters of m/z+ 180, 138 and 121 and no others. It is important to remember that the computer detection parameter is programmed in advance, so that only the appropriate daughters (180, 138, 121) if present are detected. If an alien fragment equal in mass to that of the T-2-TFA and with the same retention time is present, it will not be detected because its daughters will be different. MRM has promise of becoming the most definitive method yet developed in detection of compounds in a a biological matrix.

RESULTS AND DISCUSSION

It is helpful for purposes of discussion to present the structure of T-2-TFA and its fragments that are generated in the electron impact mode of operation. The structure of the

trifluoroacetate derivative of T-2 toxin is shown in (Figure 2). The molecular ion (M+562) is easily generated in electron impact when the source temperature is kept at 150 C. The first loss is 84 amu due to partial fragmentation of the isovaleroxy group to yield m/z+ 478. The latter is an even electron species created by retention of the ester oxygen of the C-8 substituent. Alternatively, the ester oxygen on C-8 can go with the isovaleroxy group with a loss of 101 amu and creation of the even electron species of m/z+ 461. The latter then loses an acetate group (amu 60) to generate m/z+ 401. The latter value is a key intermediate in positive chemical ionization analysis in methane and is the base peak of that reaction. The fragment m/z+ 478 however, is more useful in analyses by MRM than m/z+ 401 because the former generates the key and most stable secondary ions used in the MRM reaction. Thus, fragment 478 generates the electronically stable unsaturated rings with amu values of 180, 138 and 121. Fragment 478 can lose an acetate group (60 amu) and give rise to ion species 418 present in the spectrum. The fragment ion m/z+ 460 gives rise to m/z+ 400 present in the spectrum.

The collision voltage is an important component that will regulate the amount of collision activated decomposition of the parent molecule traveling through the first quadrupole of the instrument. The voltage was varied from 22 to 50 in increments of 4 voltage units and then in an increment of 10 volts from 50 to 60 (Figure 3). There is almost a linear increase in ion counts of amu 121, 138 and 180 in the range of 22 to 34 volts. Thereafter, amu 180 falls off and does not recover whereas amu 138 has its optimum at 38 volts and then levels

off. The greatest response for amu 121 is at 60 volts. It appears that a reasonable collision voltage recommendation is about 36-38 volts where all three daughters have an optimum intensity. Note in (Figure 2) that fragments 180, 138 and 121 are unsaturated rings. Unsaturation conveys stability to the fragments; this is a desirable trait in the selection of daughter ions.

The full mass spectrum of T-2-TFA is shown in (Figure 4-A). One should observe the intensities of m/z+ 121, 138 and 180 as they are the daughter ions of m/z+ 478 as shown in (Figure 4-B). The latter are stable, intense and the most abundant of the entire daughter ion spectrum and thus lend themselves as being ideal candidates for analysis. For purposes of contrast, the daughter fragments ions of m/z+ 460 is presented in (Figure 4-C). The daughter ion spectrum is crowded with numerous fragments the most notable of which are amu 400, 327, 227 and 120. As will be shown later, the sensitivity of this set of daughters is not as great as that of 478 and the value for the signal to noise ratio is not as high (higher noise level) as that of parent 478. It is necessary to study the secondary ions generated by all of the major parent ions of the electron impact spectrum before choosing a set of parent/daughter ions. This was done with all the major fragments in the spectrum of T-2-TFA. Fragment m/z+ 478, although low in intensity, showed the least noise level and highest sensitivity. Thus, in a matrix resulting from the extraction of blood and/or urine, it is relatively easy to find one part per billion of T-2 toxin.

The results of analysis of the trifluoroacetate derivative of T-

2 toxin is shown in (Figure 5-A) and it is compared with analysis by SIR shown in (Figures 5-B and 5-C). Urine was chosen as the biological matrix because normally it contains a more complex mixture or background than blood which is the alternative in our system. The urine was spiked at 10 part per billion and extracted as described and hence the results shown represent losses during extraction and purification of the sample. The actual amount of T-2-TFA injected is about 2 nanograms and this was extracted from 5 ml of urine. Note the low noise level of the background which tives a very high signla to noise ratio estimated to be about 100 to 1. Five ml of blood containing 10 ng of T-2 per ml was extracted and concentrated so that the final volume of 20 l contained 50 ng of T-2. One l of solution containing 2 ng T-2 was injected into the instrument. The daughter ion fragment 180 is consistantly the most dependable for quantification purposes although daughters 121 and 138 are also used.

The same extract was also analyzed by SIR as shown in (Figure 5-B and 5-C). Note the excessive noise when m/z+ fragments 478, 460 and 418 are used (Figure 5-B) and when m/z+ 180, 138 and 121 are used (Figure 5-C). Although the signal to noise ratio and thus the sensitivity of the MRM using 478/180,138,121 parent daughter set is very high in MRM, these same amu fragments used in SIR yield equivocal results and are less sensitive.

The parent ions m/z 478 and 460 are compared for sensitivity in (Figure 6-A and 6-B). The same extract of blood amended with T-2 toxin at a concentration of 10 parts per billion was used for comparison and it is readily seen that the parent/daughter set of 478/180, 138, 121

is more sensitive than the 460/400, 327,227 set. Compare the daughter ion spectra of these sets in (Figure 6-A and B). The signal to noise ratio of the 478 parent/daughter set is clearly about 400:1 as compared to the 460 set at approximately 100 :1. Both parents have been analyzed by Multiple Reaction Monitoring.

To obtain the maximum sensitivity from MRM, we recommended that the sectoring portion (magnet) of the instrument operate at a resolution of about 1,000 to 2000. It is possible to pass the parent fragment through the magnet with a resolution of 10,000 or greater; however, in doing so, sensitivity is sacrificed although accuracy of transmission of the desired species is assured. However, it is possible at a resolution of 1000 that an alien fragment having the same amu value as the one chosen (example amu 478) will have the same retention time as that of T-2-TFA. Thus in (Figure 7), the analysis of T-2 toxin in blood is shown using the parent amu 478. Note that at a retention time near to but not identical to that of T-2 toxin, an interfering substance can be seen with an intensity greater than that of the T-2 toxin (Figure 7-A). The ion fragments measured by the last quadrupole were 478 (parent) and 121, 138, 180 (daughters of T-2). This means that m/z+ 478 parent was allowed through the first half of the instrument, into the collision chamber of quad #1 and into the analyzer of quad #2. However, as seen in (Figure 7-B), the daughter ions of the alien 478 are 141, 168, 197, 251, and 364, clearly different from that of T-2-TFA. Therefore when compared with the daughter ion spectra of the trichothecene library, no match was generated whereas with T-2-TFA, a good library identification was

established.

Multiple reaction monitoring was reduced to practice by ammending whole blood (human) with T-2 toxin at 1, 10 and 100 parts per billion. The extraction procedure involved precipitation of the heparinized blood with acetone, centrifugation to eliminate the precipitate and finally passing the extract through a 500 mg Bondelute column. The column was washed with 2 ml of water followed with elution with 2 ml of acetone. The eluant was concentrated and reacted with trifluoroacetic acid anhydride and injected into the gas chromatograph of the tandem mass spectrometer.

As shown in Figure 8, there was a linear relationship between 1, 10 and 100 parts per billion when analyzed by MRM. The percent recovery is not known because of the unavailability of a suitable internal standard. However, the T-2 toxin was added to the blood before extraction and hence represents the amount recovered after the entire analytical procedure. The signal to noise ratio was such that significant numbers were generated that allowed facile quantification at 1 ppb.

The uniqueness of tanden mass spectrometry and multiple reaction monitoring is such that accuracy in identification and sensitivity in detection is very high. Although the precision based on three replicates is acceptable, it will improve when an internal standard is available. To this end, we are synthesizing C4-tridueterated acetate of T-2 which will increase our precision in quantification and perhaps increase our sensitivity.

Figure 1. Block diagram of a hybrid GC/MS/MS system. The first mass spectrometer (MS1) is comprised of the electrostatic analyzer (ESA) a magnet with an intermediate detector. This is the sectoring portion of the instrument. The quadrupole portion of the mass spectrometer is next in line and is used as the collision cell where collision activated decomposition occurs (daughter ions are formed). The final mass spectrometer is quadrupole number 2 and it functions as a mass scanning detector. The samples to be analyzed are resolved on a gas chromatograph before entering the mass spectrometer.

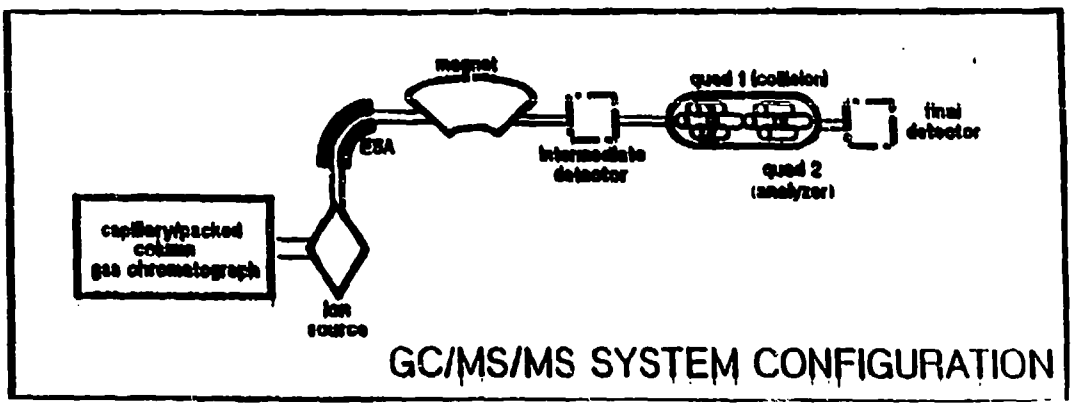


Figure 2. Fragmentation pathway for the trifluoroacetate derivative of T-2 toxin. Fragment amu 478 (parent) gives rise to fragments (daughters) 180, 138 and 121. The double bonds in the aromatic ring distributes the ionic charge thus causing the production of very stable fragment species.

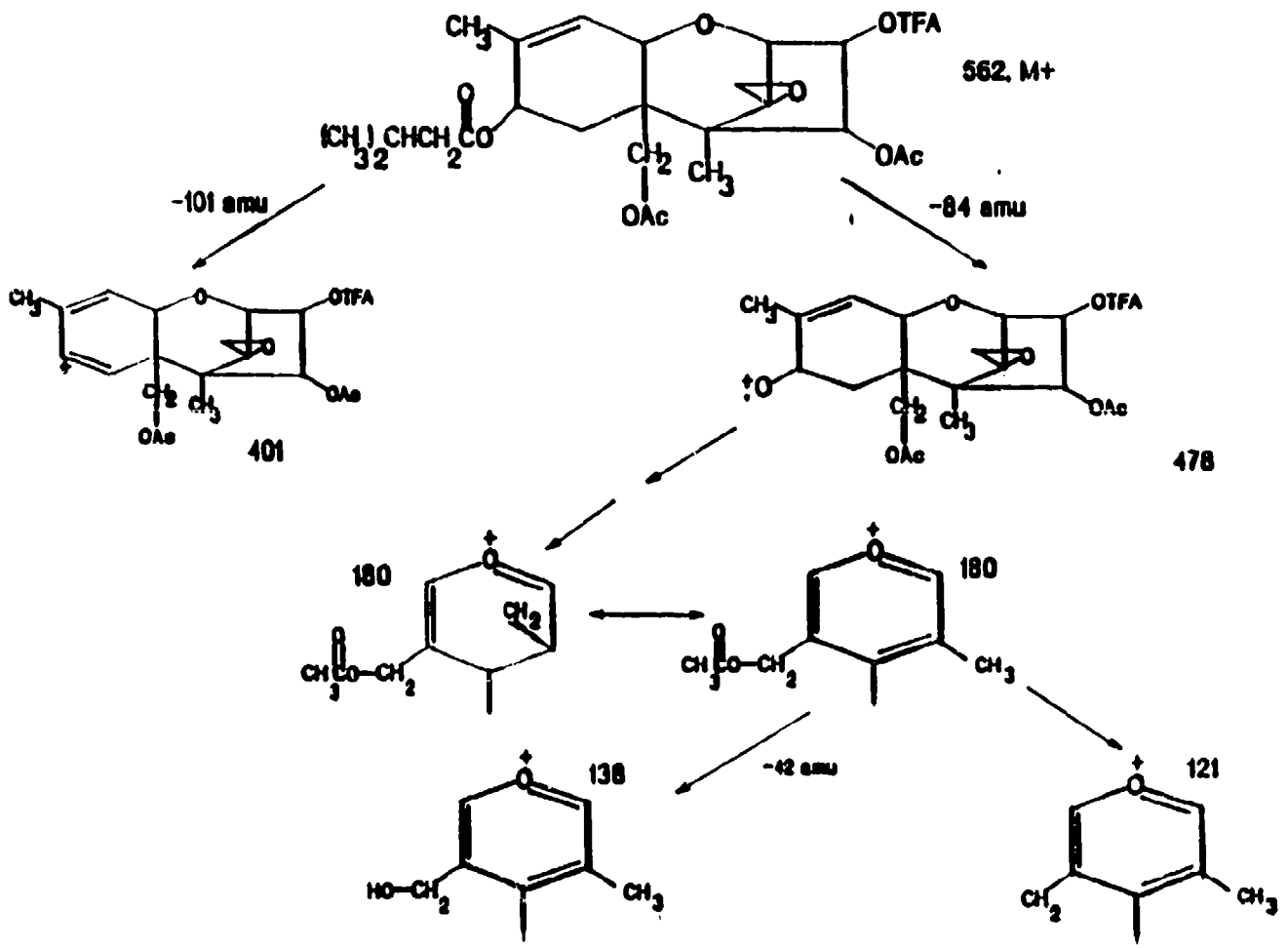


Figure 3. Line graph of experiments showing the results of variable collision energy (22-60 volts) and ion abundance of daughters generated from the parent fragment of 478. A collision voltage of 34-40 volts produced optimal intensities for all three daughters 121, 138 and 180.

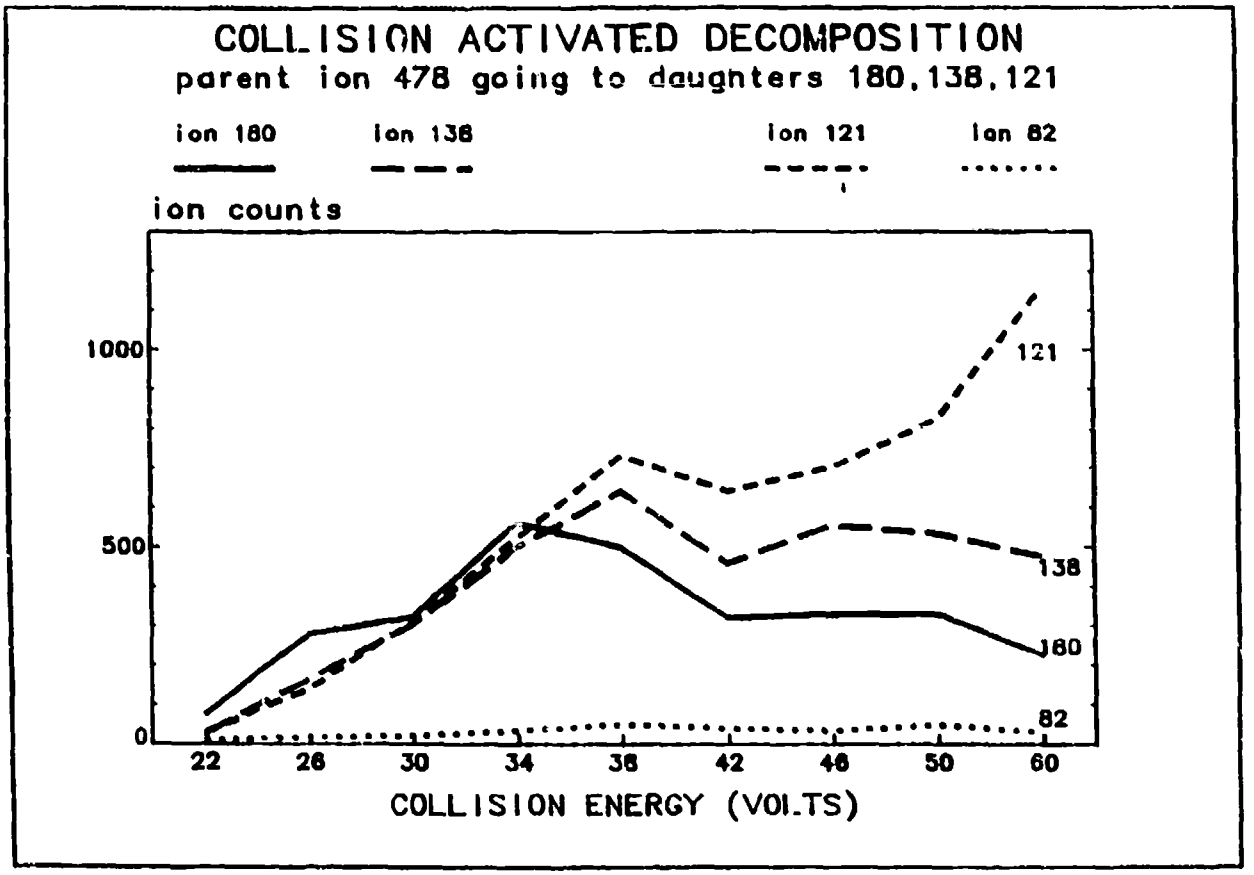


Figure 4. (A) Full scan electron impact mass spectrum of the trifluoroacetate derivative of T-2 toxin. The inset shows daughters generated from parent fragments 562 and 478. (B) Daughter ion fragments generated from parent amu 478. (C) Daughter ion fragments generated from parent amu 460. The base peak and those fragments with greatest intensity found in the full scan mass spectrum (121, 138 and 180) results from the parent 478. Parent amu 460 produces a mixture of less outstanding daughter ions.

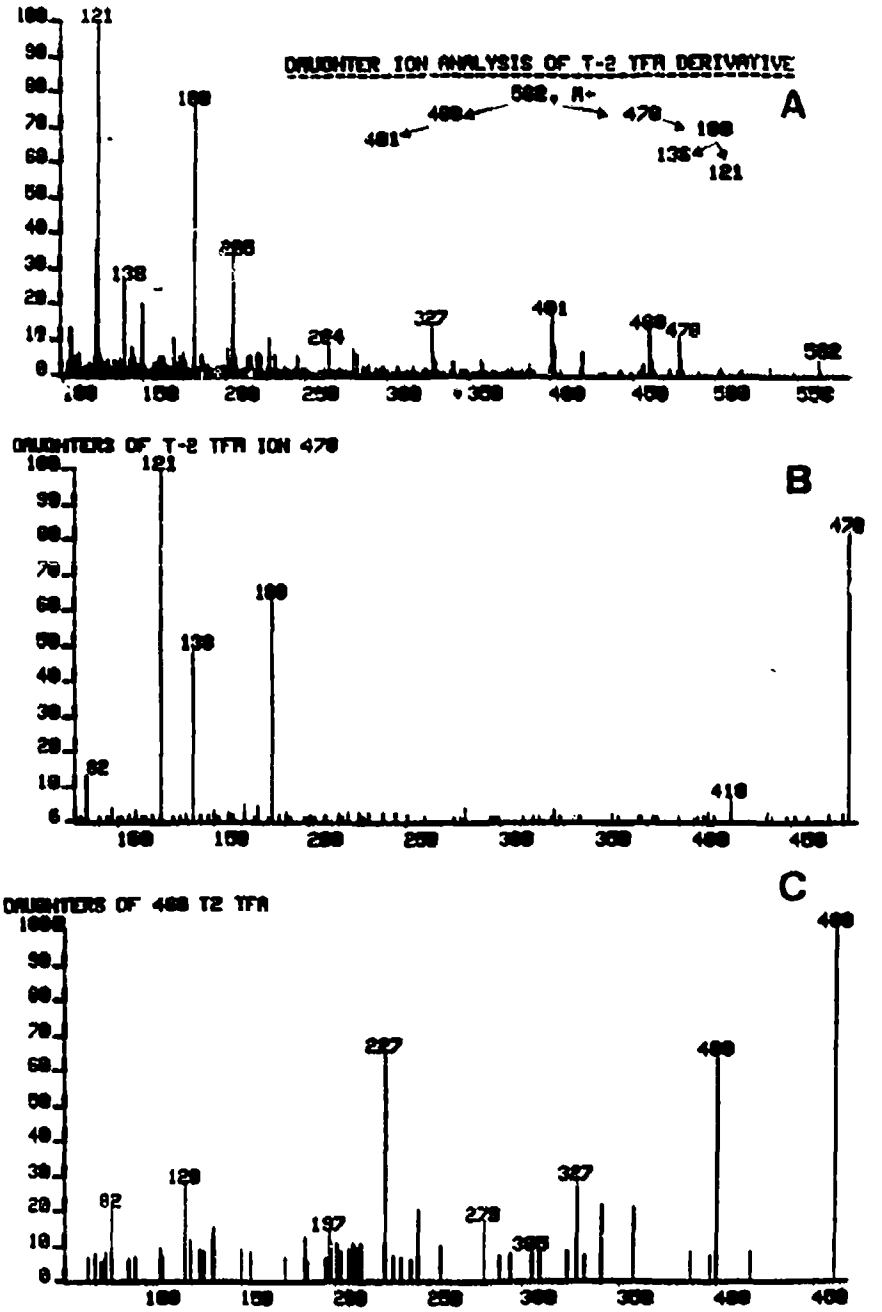


Figure 5. Comparison of the analysis of T-2 toxin in human urine by multiple reaction monitoring (MRM) and selected ion recording (SIR). (A) Analysis by MRM using the parent 478 and measuring the daughters 180, 138 and 121. Note the minimum amount of noise (base line) from the biological matrix. (B) Analysis by SIR for three ions 478, 460 and 418 and (C) analysis by SIR for three different ions, 180, 138 and 121. Signal to noise ratio for SIR is about 3:1 and for MRM about 20:1.

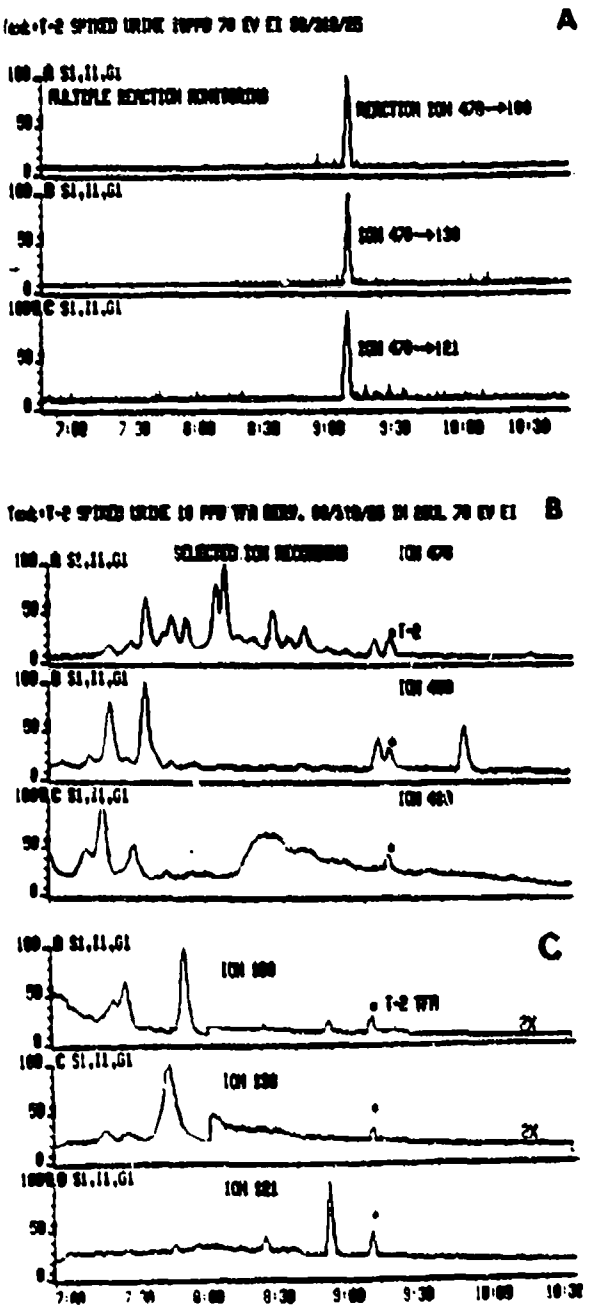


Figure 6. A comparison of two MRM experiments (parent 478 vs 460) in the analysis of T-2 toxin in human blood. (A) Parent 460 was selected and corresponding daughters 400, 327 and 227 were measured. (B) Parent 478 was selected and the corresponding daughters 180, 138 and 121 were measured. The greatest sensitivity and signal to noise ratio was found with m/z+478. Note the quiet base line in B.

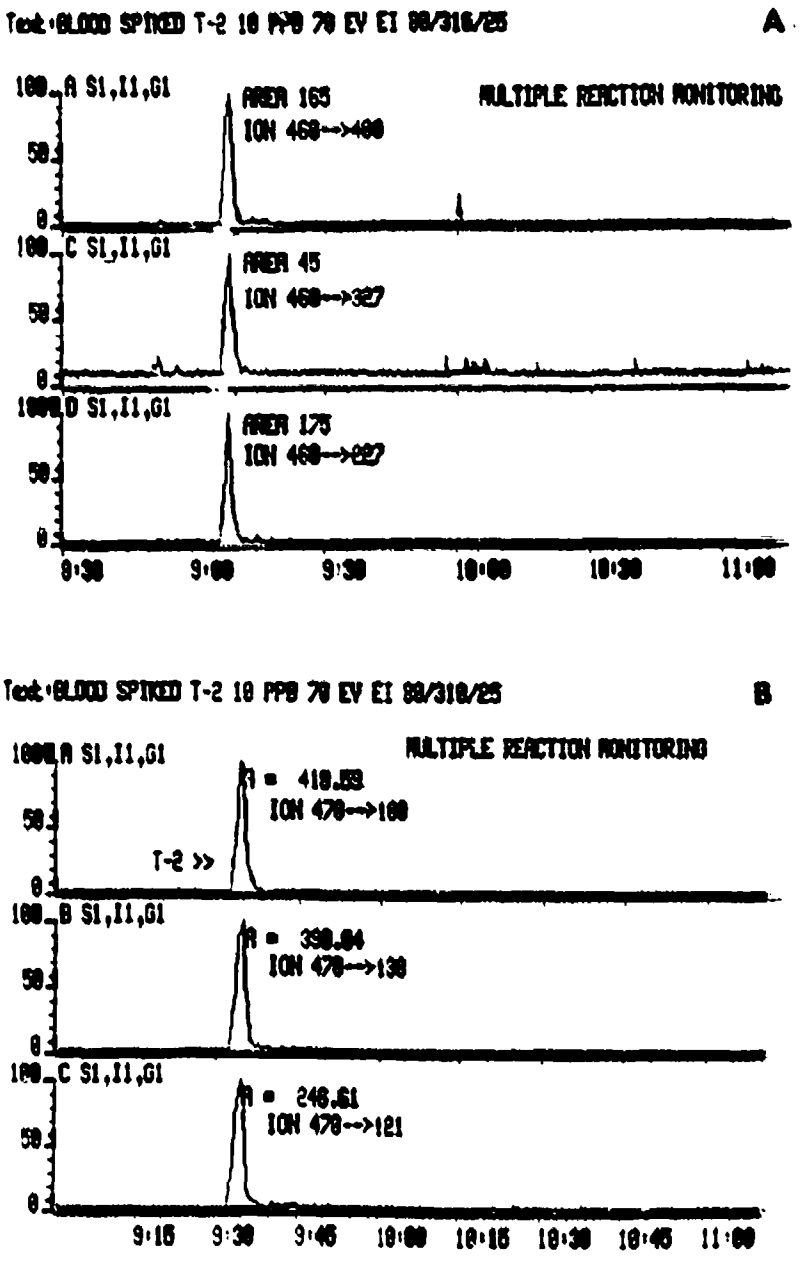


Figure 7. Interfering substance found in human blood when analyzed by MRM using m/z+478 as the parent ion. Boxes A and B both show the total ion chromatogram of fragment 478 as detected by quadrupole #2. The retention time of the unknown component is different from that of T-2. However, when a library search is made of the daughters generated by the unknown component, there is no match as the daughters of the unknown are completely different i.e. 180, 138 and 121 for T-2 versus 364, 251, 197 and 141 for the unknown.

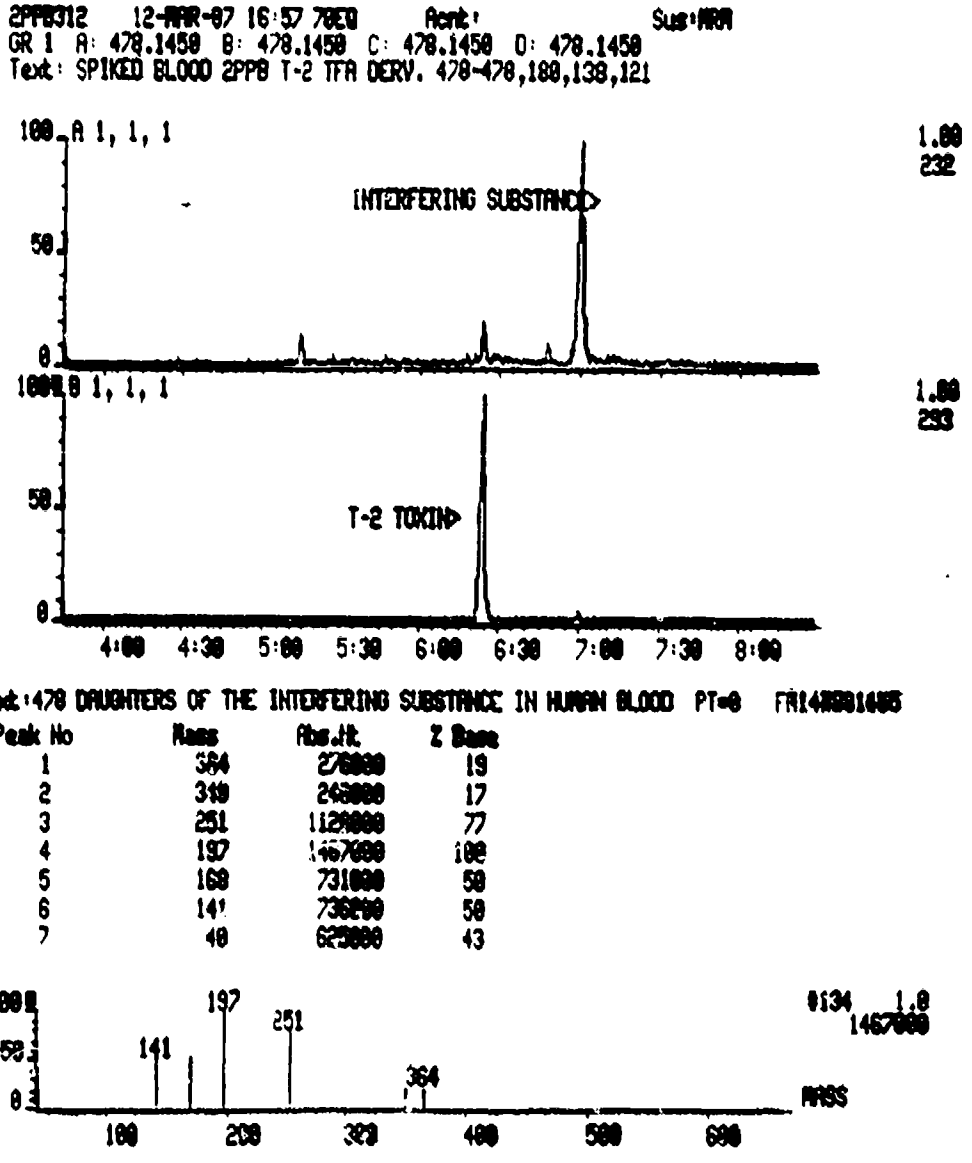
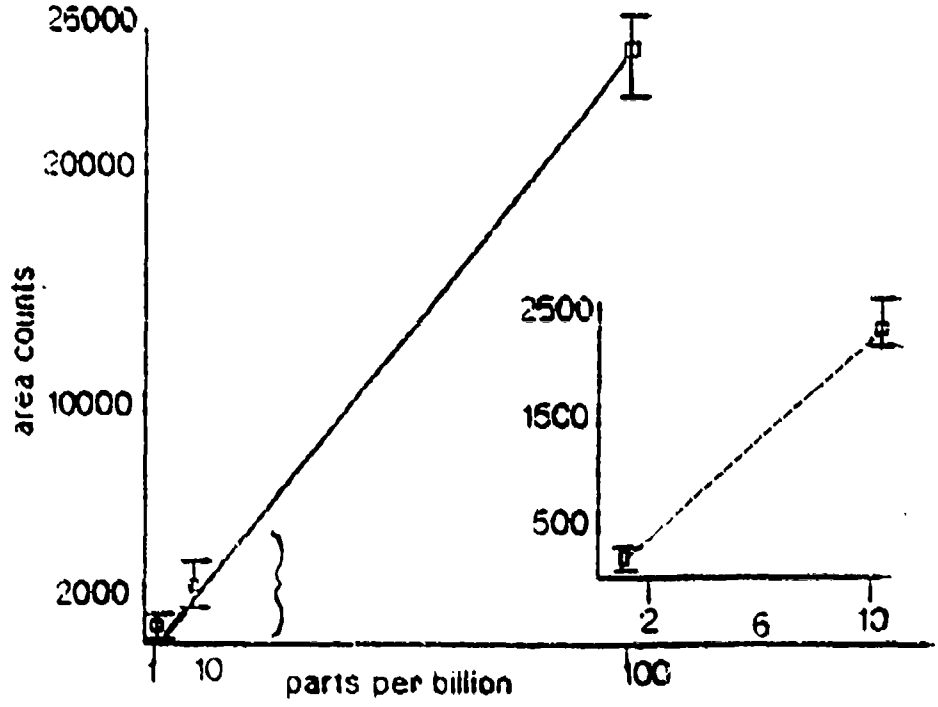


Figure 8. Extraction and detection of T-2 toxin in whole human blood (amended so as to give concentrations of 1, 10 and 100 parts per billion) using multiple reaction monitoring. The results are based on the average of three replications and are plotted using the least squares method. The inset gives the variation around the 1 and 10 ppb points.



ANALYSIS OF T-2 TOXIN IN HUMAN WHOLE BLOOD USING DEUTERATED INTERNAL STANDARDS

Samples of whole blood were amended with T-2 toxin at concentrations of 1, 10 and 20 parts per billion as well as 1, 3 and 5 ppb. The blood samples were also amended with a deuterated standard of T-2 in order to improve quantitation and measure recovery. T-2 was deuterated in the C-3-acetoxy position as described in the last section of this report. The standards were placed in the blood samples before extraction so they reflect total recovery of the method.

The method used in this analysis is described in the previous section entitled Use of Multiple Reaction Monitoring in Tandem GC/MS/MS. Essentially, the sectoring portion of the instrument (VG7070EQ) is locked on to masses 478 for the T-2-TFA toxin and on 481 for the T-2-TFA deuterated standard. Quadrupole number 1 acts as the reaction chamber where the nonionized argon gas reacts with the ionized 478 and 481 ionized fragments of T-2-TFA. A voltage potential varying between 28 to 38 volts is placed on the reactants in Quad #1; this is called collision activated decomposition. The nonionized argon acts as the collision gas and the electrons provide the initial energy for decomposition (CAD). CAD is unlike chemical ionization in that the latter process utilizes an ionized gas and the molecule being measured is nonionized. Quadrupole #2 acts as the detector of the daughter ions and is being scanned for only the daughter ion fragments in question. As an example, the nondeuterated T-2-TFA is set up so that m/z+ 478 will detect only daughters with an amu value of 180, 138 and 121. Likewise, the deuterated T-2-TFA standard is so set up so that

daughters 180, 138 and 121 from m/z+ 481 (478 + 3d) are detected. Although the daughters from 478 and 481 are identical in mass value, the reporting (quantitation) is done in different channels so that daughters generated from 478 do not interfere from daughters generated from 481.

ANALYSIS OF WHOLE BLOOD AT 1, 10 AND 20 PARTS PER BILLION

The results of this experiment is summarized in (Figure 9) which shows the quantitation of T-2 at 1, 10 and 20 ppb based on an internal standard. The precision of three determinations at 1 and 10 ppb is very good with only one outlier at 20 ppb. Similar results were obtained when T-2-TFA was used as an external standard (Figure 10). The precision of the latter at 1 and 10 ppb is good with some scatter at 20 ppb.

The analyses were made using a VG7070EQ tandem, hybrid mass spectrometer in the multiple reaction monitoring mode. The daughters monitored were m/z+ 121 138 and 180; these were generated from the parent fragment m/z+ 478. The equivalent parent from the deuterated standard was m/z+ 481 (478 + 3d).

The results of the analyses are shown in both Table 1 and (Figures 9 and 10). As shown in Table 1, calculations were made on both daughter ions 121 and 180 based on the values obtained from the deuterated standard. Calculations based on daughter 180 of the standard averaged 16 ppb for the 20 ppb amended sample, 7.2 for the 10 and 0.7 for the 1.0 ppb. The values seem reasonable and have been accepted without further modification for percent recovery.

Figures 9 and 10 show the plot of the recovery of 1, 10 and 20

ppb amended samples. The precision at 1 and 10 ppb is excellent whereas at the 20 ppb level it has a moderate spread but is acceptable. The slope of the graph plotted from the values obtained from the external standard is slightly steeper but not significant. The two graphs are almost identical showing excellent precision at 1 and 10 ppb with moderate spread at 20 ppb.

The method claims sensitivity down to one part per billion in a biological (blood) matrix. When 1 ppb is added to whole blood and then extracted, purified, the derivative made and then injected into the GC/MS/MS, T-2-TFA can be detected easily as shown in Figure 11. The area count as indicated is 9 and the signal to noise ratio immediately preceding and after the peak is about 100:1.

The advantage in the use of deuterated T-2-TFA standard can be seen in Figure 12. Channels A and B show the analysis of daughters 180 and 121 from the analyte (T-2-TFA). Channel C shows daughter 180 arising from the internal standard and with a parent of 481 i.e. 478 plus 3. Although the 180 daughter has the same numerical value as 180 arising from the analyte i.e. the T-2 toxin being analyzed, the two values are not confused because they are kept in separate channels. Thus, quantitation is neatly packaged so that the two values are not confused. Moreover, the values are so close, that there is little opportunity for error.

Table 1. Quantitation of T-2-TFA toxin recovered from blood amended with T-2 to give final concentrations of 1, 10 and 20 parts per billion. Area counts of daughters are given for m/z+ 478 going to 180 and m/z+ 481 of the deuterated standard going to daughter 180. Area counts, standard deviations and averages are presented. The standard used was T-2-TFA deuterated at the C-4-trideutero-acetate-T-2-TFA.

Concentration T-2 ppb	Area Count 478-->180	ISTD 481-->180	ppb	SD
20	73	28	24	11.2
20	37	20	11	
20	49	31	12	
Average			16	
10	41	29	7.5	1.2
10	33	17	7.0	
10	41	28	7.5	
Average			7.2	
1	9	8	0.8	1.0
1	5	4	0.8	
1	4	8	0.5	
Average			0.7	

Figure 9. Plot of the relationship of 1, 10, and 20 ppb of T-2 toxin in blood when extracted, subjected to column purification, reacted with trifluoroacetic acid anhydride and resolved on a capillary gas chromatography column (GC/MS/MS). The method of analysis used was MRM on a VG7070EQ. The quantitation was based on an internal deuterated standard.

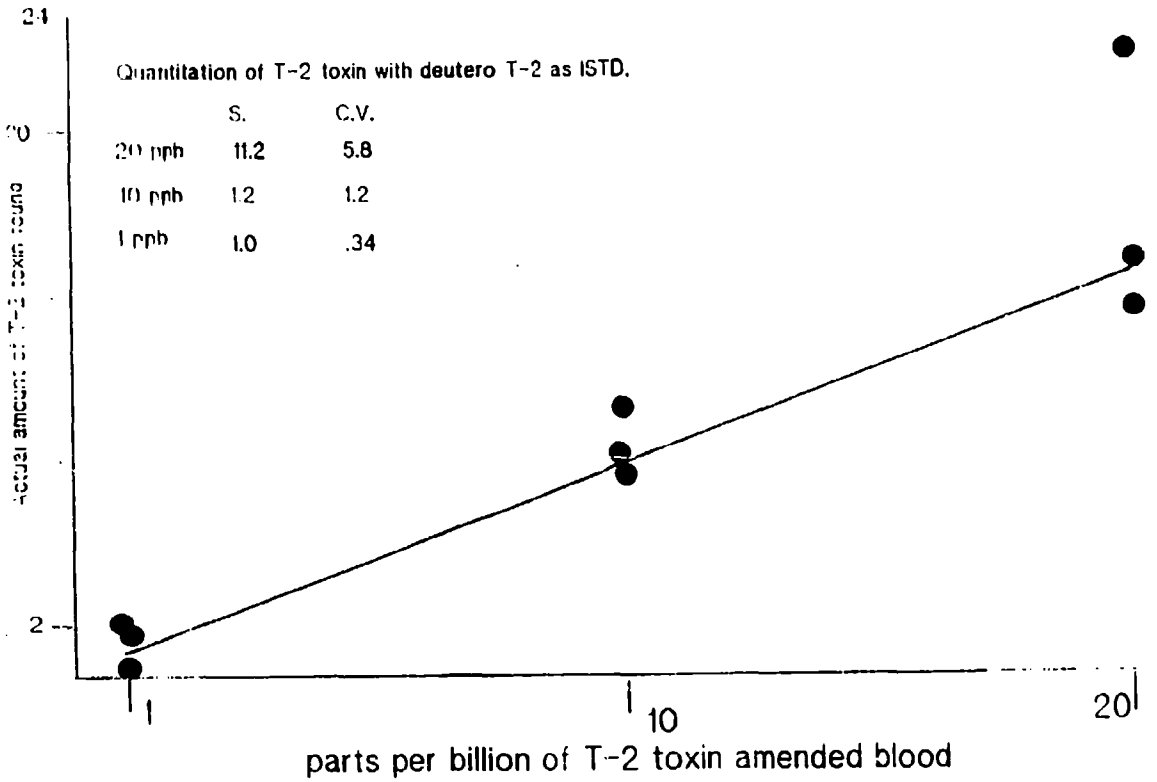


Figure 10. Plot of T-2-TFA after extraction, purification and resolution by GC/MS/MS as in Figure 1 except that quantitation is based on an external standard.

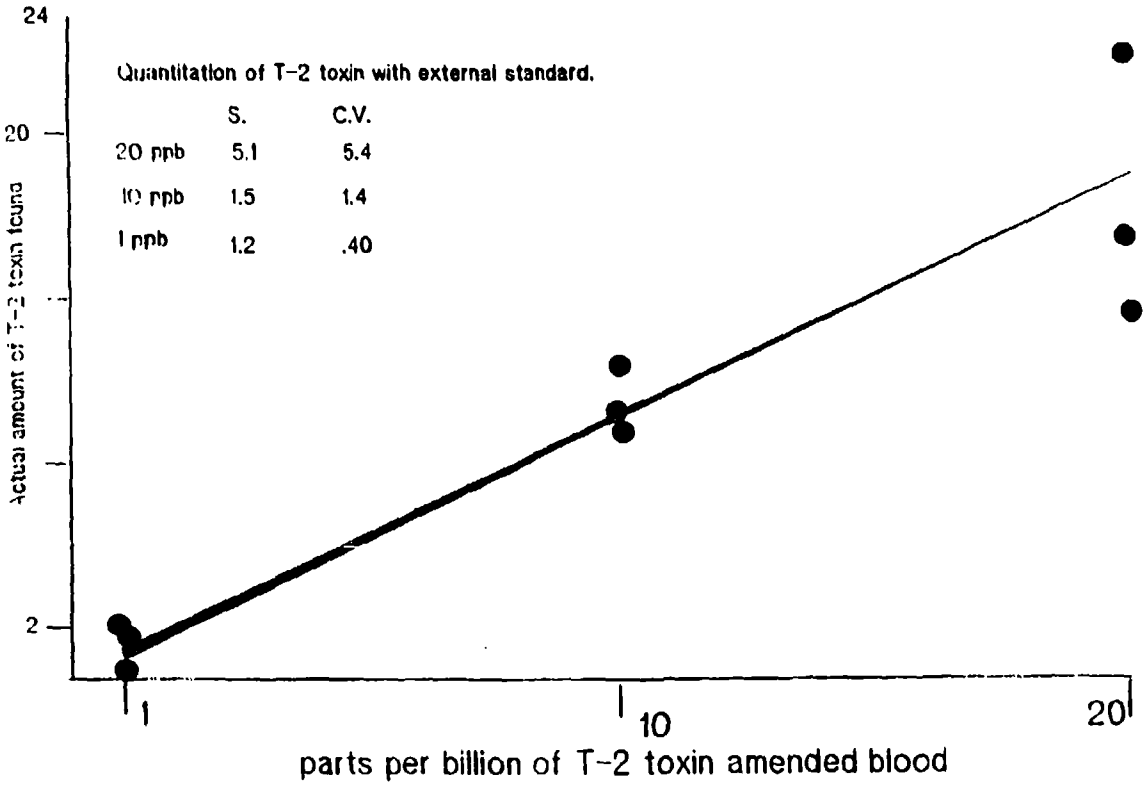


Figure 11. Total ion chromatogram of the daughter ion (m/z+180) generated from the parent m/z+481 of the deuterated standard. The analysis represents that of T-2 toxin amended into whole human blood to give a final concentration of 1 part per billion.

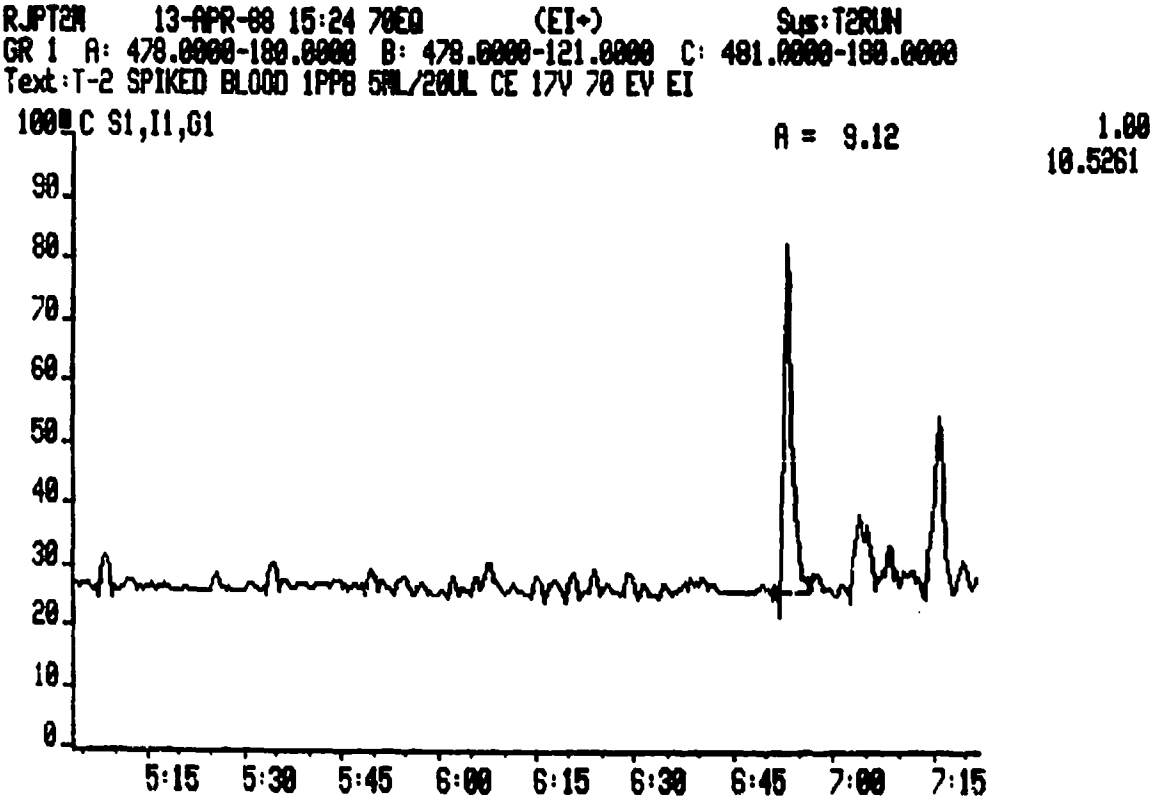
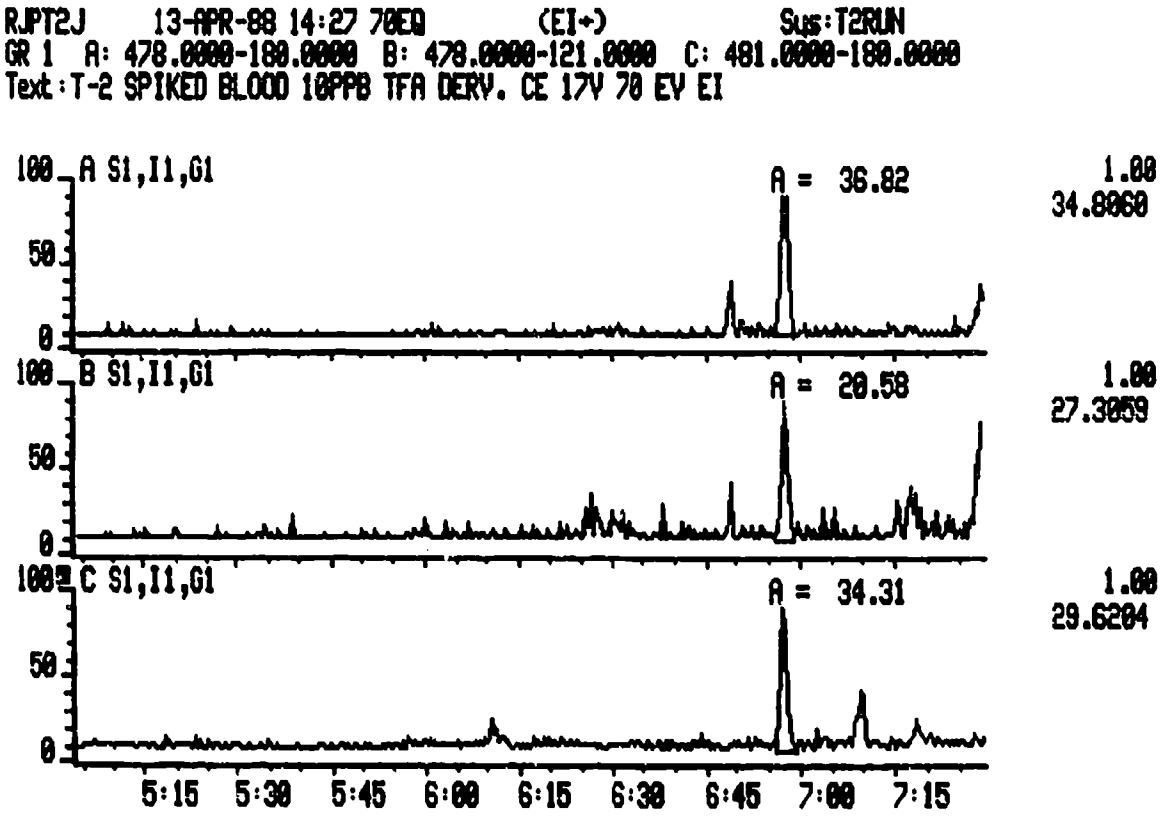


Figure 12. Total ion chromatogram of T-2 toxin amended into whole blood to give a concentration of 10 ppb. The parent ion monitored was 478 and the daughters shown are 180 and 121. Channel C shows the daughter (180) generated from the deuterated T-2 toxin using the parent m/z+481. Although both 478 and 481 yield the same daughters (121, 138 and 180) they do so in different channels. This means that the generation of 180 from 478 does not interfere with the generation of 180 from 481.



ANALYSES OF WHOLE BLOOD AT 1, 3 AND 5 PARTS PER BILLION

The experiment was repeated using lower concentrations of T-2 toxin in order to verify the results at the lower concentrations (1 and 3 ppb) and to show the reproducibility of the method. Moreover, we thought it important to repeat the experiment at low concentrations immediately after replacing the electron multiplier. Experiment #1 (1, 10, 20 ppb) was run with an electron multiplier that was not at optimum. The results of the analyses are shown in the next page on Table 2.

The precision of the counts at each level appeared to be very good; however, the accuracy may not have been at optimum. The relative values gave a good straight line relationship (Figure 16) but they were high. As an exmaple, samples amended with 5 ppb gave an average of 9.4 ppb, the 3 ppb amendment, gave 5.2 ppb and the 1 ppb was 2.2 ppb. Although analytically, the results are acceptable in so far that the precision is good but the accuracy is off by about 48%.

The cause of the disparity may be multiple: (1) error in weighing out the standards either the nondeuterated T-2 or the deuterated T-2 (2) there may be specific enhancement effects on the part of the nondeuterated T-2 relative to the deuterated form. Enhancement effects are well known in mass spectrometry. It is more reasonable to suspect that the deuterated-T-2 toxin standards may be in error because the quantity of standard is minimal and this precludes accurate weighing of the primary standard.

The plot of the actual values obtained for the amended blood samples (1,3,5 ppb) are shown in (Figure 16). The precision at 1 ppb

is excellent whereas at 3 ppb there is about a 17% spread. The highest concentration (5 ppb) usually has the greatest scatter (41%) and this experiment was no exception. We are impressed with the precision of the replications within the treatments; although the accuracy is about 50%, at these low parts per billion values, we find them acceptable.

Figures 13, 14 and 15 show the actual total ion current chromatograms of the multiple reaction monitoring runs. All of the quantitation done was done with daughter 180 of m/z+ 478 and m/z+ 481. The signal to noise ratio for the 3 and 5 ppb concentrations are excellent. The one ppb level shows more noise than the others because of the low counts obtained relative to the higher concentrations. The signal to noise ratio for the T-2 toxin is excellent at 1 ppb whereas the noise in the deuterated standard is on the level of good. For some reason the deuterated standard is noisier than the non-labeled T-2 toxin.

A linear plot of the relationship between area counts of 1, 3, and 5 parts per billion concentrations is shown in Figure 16. In this experiment, the blood was amended with T-2 toxin and then subjected to the complete method of analysis which included, extraction, cleanup on a column etc. The results so obtained are encouraging as there is good agreement in the values obtained i.e. they follow a straight line although the values for recovery are high.

Table 2. Quantitation of T-2-TFA toxin recovered from blood amended with T-2 to give final concentrations of 1, 3 and 5 parts per billion. Area counts of daughters are given for m/z+ 478 going to 180 and m/z+ 481 of the deuterated standard going to daughter 180. Area counts, standard deviations and averages are presented. The standard used was T-2-TFA deuterated at the C-4-trideutero-acetate-T-2-TFA.

	Concentration T-2 ppb	Area Count 478-->180	ISTD 481-->180	ppb	SD
	5	87	44	9.8	1.97
	5	116	56	10.4	
	5	146	91	8.02	
Average		116	64	9.4	
	3	78	51	4.5	1.8
	3	79	51	4.6	
	3	65	30	6.5	
Average		74	44	5.2	
	1	46	21	2.2	0.5
	1	42	22	1.9	
	1	31	12	2.6	
Average		40	18	2.2	

Figure 13. Resolution and analysis by MRM of T-2 toxin amended into whole blood so as to give a concentration of 5 ppb. Channel E shows the total count for the 478 parent and 180 daughter of T-2 whereas channel D shows the 481 parent of the deuterated standard going to the 180 daughter. FIVE PARTS PER BILLION

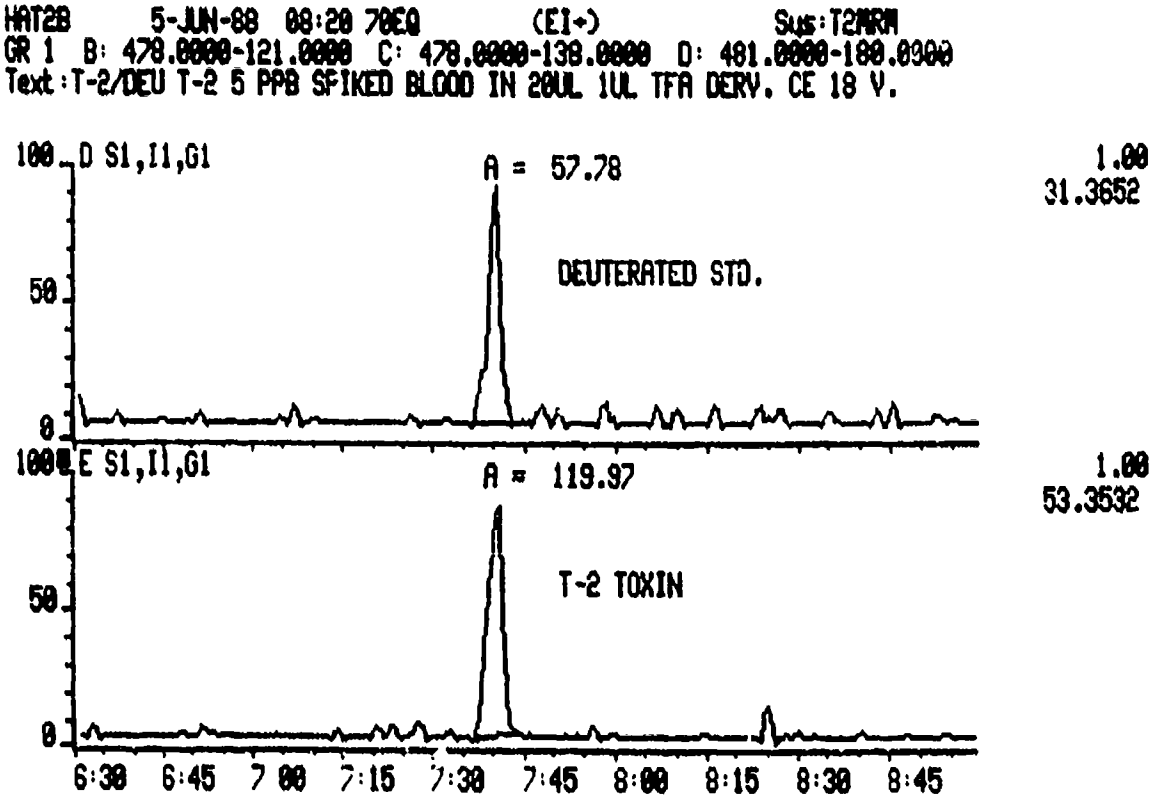


Figure 14. Resolution and analysis by MRM of T-2 toxin amended into whole blood so as to give a concentration of 3 ppb. Channel E shows the total count for the 478 parent and 180 daughter of T-2 whereas channel D shows the 481 parent of the deuterated standard going to the 180 daughter. THREE PARTS PER BILLION

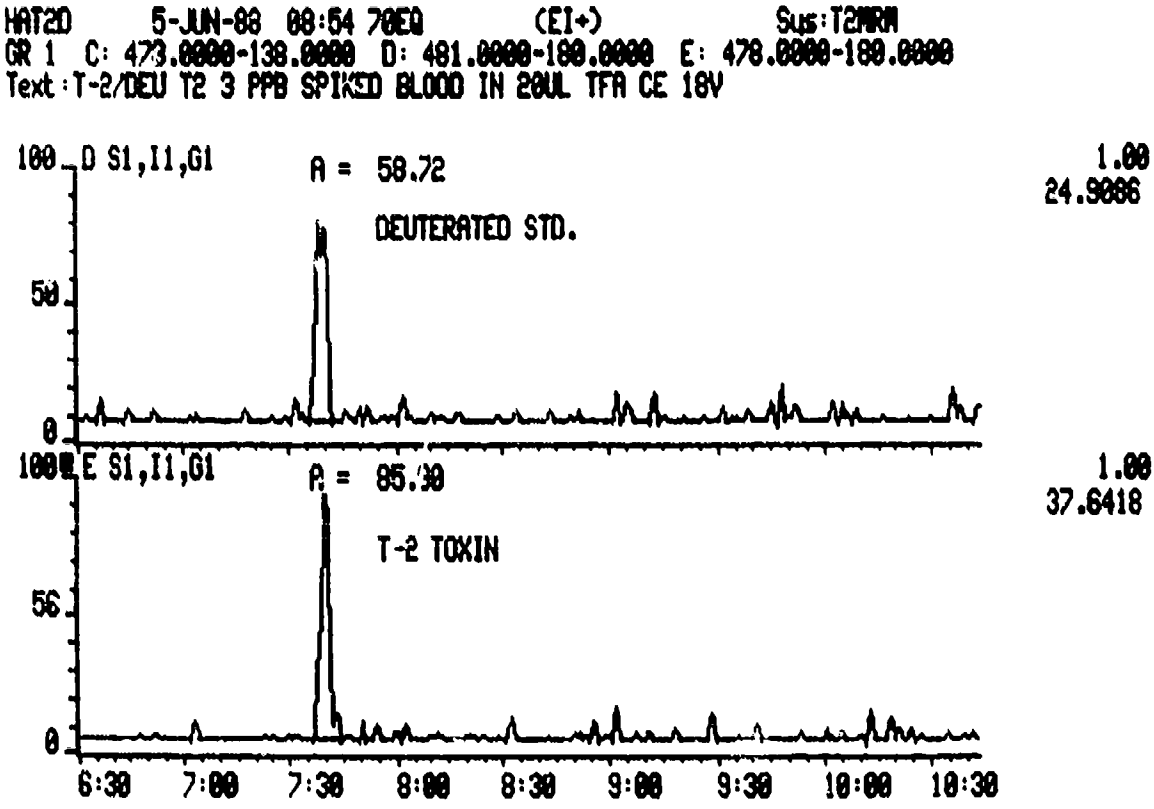


Figure 15. Resolution and analysis by MRM of T-2 toxin amended into whole blood so as to give a concentration of 1 ppb. Channel E shows the total count for the 478 parent and 180 daughter of T-2 whereas channel D shows the 481 parent of the deuterated standard going to the 180 daughter. ONE PART PER BILLION

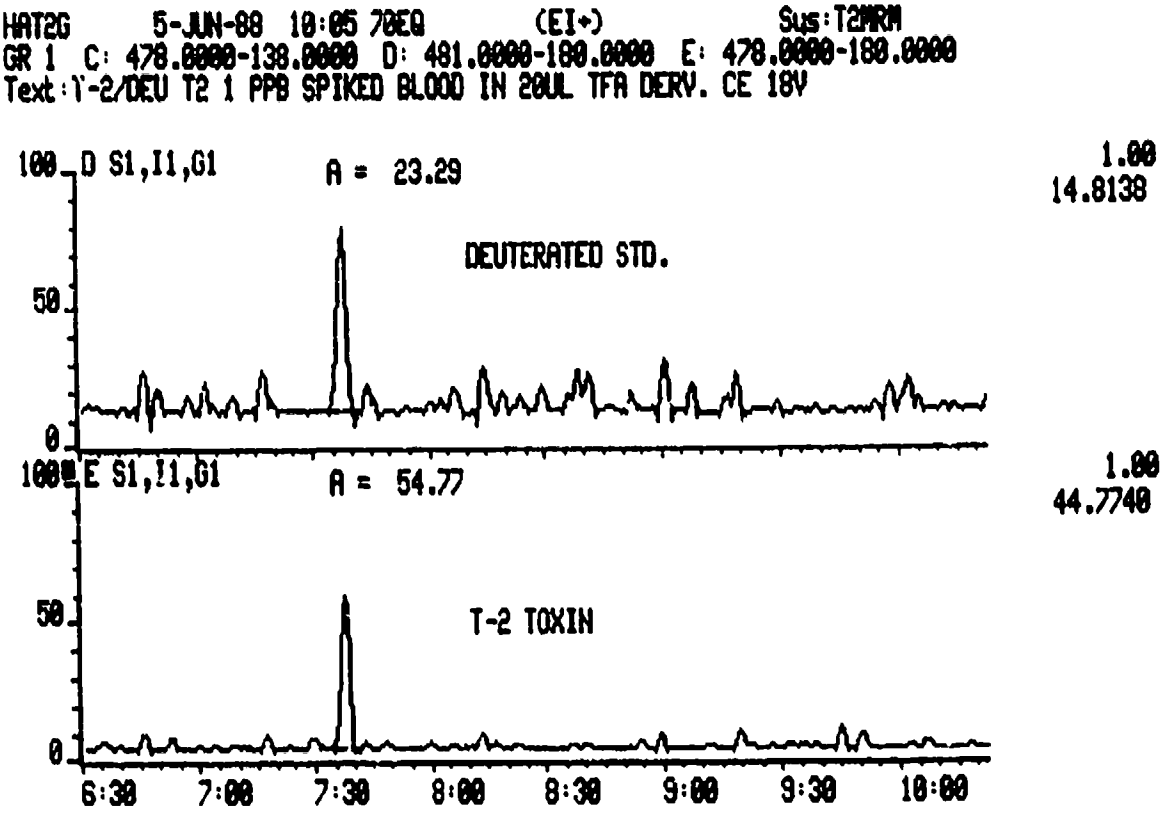
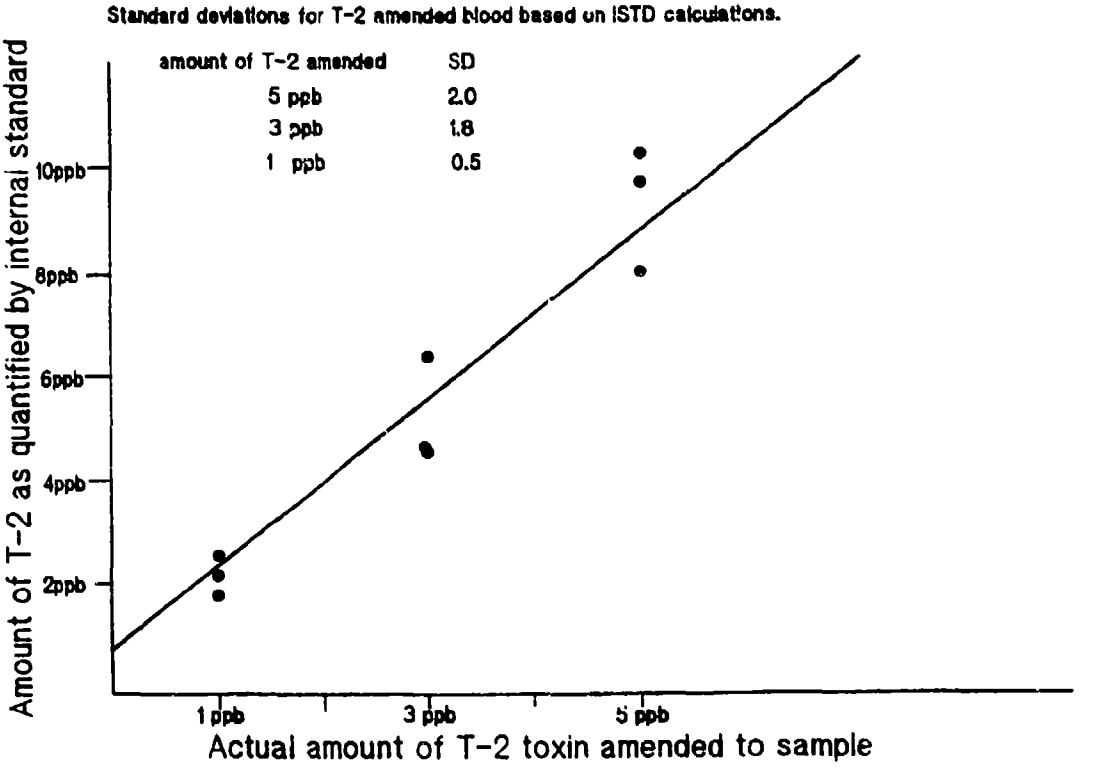


Figure 16. Plot of the relationship of 1, 3, and 5 ppb of T-2 toxin in blood when extracted, subjected to column purification, reacted with trifluoroacetic acid anhydride and resolved on a capillary gas chromatography column (GC/MS/MS). The method of analysis used was MRM on a VG7070EQ. The quantitation was based on a deuterated internal standard using daughter 180.



ANALYSIS OF HT-2 TOXIN IN A BIOLOGICAL MATRIX USING MULTIPLE REACTION MONITORING (MRM)

Daughter Ions of 532:

The electron impact mass spectrum of the deuterated HT-2 standard is presented in Figure 17. The molecular ion is 532 plus 3 to give 535. Parents 532 and 535 respectively yield daughters 180, 138 and 121. These ions are daughters of parent 532. Since these are high abundance ions and are formed from a significant precursor of the molecules intact skeleton, they are an excellent choice for MRM analysis.

Quantitation of HT-2 Toxin:

The trifluoroacetyl derivatives of HT-2 and deuterated HT-2 were resolved on a capillary column and analyzed. The electron impact mass spectrum of the deuterated (15-trideuteroacetyl-HT-2) is shown in (Figure 17). Mass fragments 183, 535, and 619 differ from HT-2-TFA by 3 atomic mass units. Channels B, C and E (Figure 18) show the TIC derived from the reactions of parent ion 532 giving rise to daughters 138 and 121 whereas parent ion 535 generates the same daughters but in separate reactions. The calculations were made on the integrated areas of Channel E (deuterated HT-2 standard) and compared with Channel C of HT-2 (blood amended with HT-2 at 20 ppb). The plot of the quantitation using the deuterated internal standard is shown in (Figure 19). The accuracy and precision found at 1 and 10 ppb is superior to that at 20 ppb with an approximate disparity between replicates of 50%. The HT-2 plot based on the external plot showed excellent accuracy and precision at 1 and 20 ppb with a 30% disparity at 10 ppb. The percent

recovery at 20, 10 and 1 ppb when calculated on the basis of the deuterated internal standard is 96, 90, and 140 respectively (Table 3). If the 2 of 3 best data points for 1 ppb is taken, then the percent recovery is 70%.

The trifluoroacetyl derivative of T-2 toxin yields ions 461 and 478 in the 70 eV, electron impact mass spectrum. The former ion is made when the entire isovaleroxy group on C-8 is cleaved whereas 478 is favored after partial cleavage of the C-8 isovaleroxy leaving an intact hydroxyl group. Both ions are formed in equal abundance except that m/z+ 478 goes on to form intense and stable secondary ions at 180, 138 and 121. It is these three reactions that account for the sensitivity (1 ppb) of the method. The trifluoroacetyl derivative of HT-2 also yields the same secondary ions at T-2 but the parent ion (532) is different. The deuterated TFA derivative of HT-2 fragmented to daughters 183, 139 and 121 indicating that the deuterium labeled acetate on C-15 remains attached to the intact and unsaturated A ring. Unsaturation of the A ring promotes stability. The deuterated T-2-TFA derivative has a deuterated acetate on C-4; hence, there is no chance of the deuterated label being found on the 180 and 138 daughters. The deuterated standards indicate that the constituents on C-15 remain attached to the A ring of both T-2 and HT-2 toxins.

Table 3. Quantitation of HT-2-TFA toxin recovered from blood amended with HT-2 to give final concentrations of 1, 10, and 20 parts per billion. Area counts of MRM selections are given. Calculated quantities, averages, and standard deviations are given. The ISTD was HT-2-TFA deuterated at the C-15 position.

Concentration HT-2 ppb	Area Count HT-2-TFA 532--->180	Area Count deu-HT-2-TFA 535--->183	ppb	SD
20	135	206	13.1	4.7
20	149	146	20.1	
20	187	211	25.2	
Average			19.3	
10	76	66	8.9	2.8
10	85	76	8.9	
10	104	109	9.2	
Average			9.0	
1	45	80	0.6	.96
1	32	17	2.1	
1	60	72	0.8	
Average			1.4	

Figure 17. Electron impact mass spectrum of the trifluoroacetyl derivative of 15-trideuteracetyl-HT-2 toxin. Note that m/z+ 532 in the normal HT-2-TFA is now m/z+ 535.

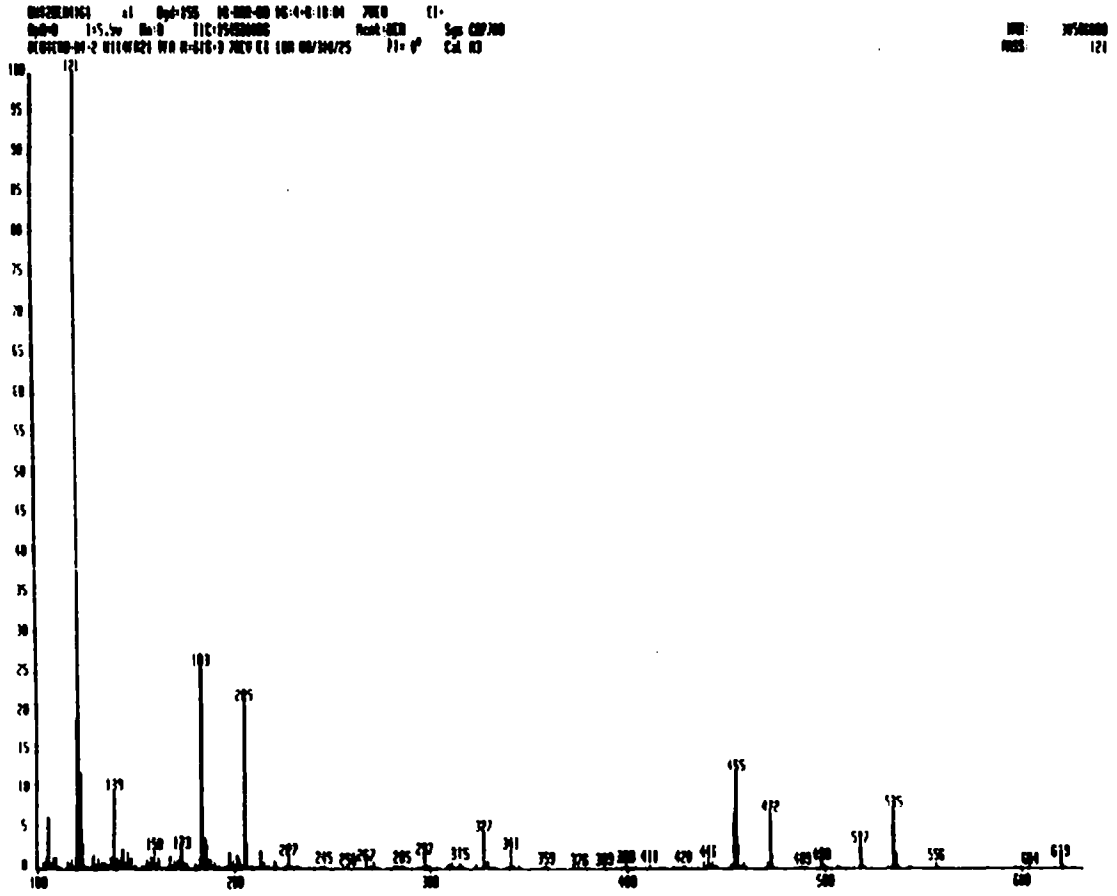


Figure 18. Total ion tracing of selected reactions obtained from multiple reaction monitoring of HT-2-TFA and its deuterated derivative. Channel B shows the results of parent 532 of HT-2-TFA going to its daughter m/z+138 and channel C of 532 going to m/z+ 121. Channel E contains the results of the deuterated standard (parent 535 going to daughter 121). The concentration of amended HT-2 in the blood sample is 20 ppb (ng/ml). Note the low noise levels in the background due to biological matrix affects. Note that although daughter ion 121 is common to both HT-2-TFA and deuterated HT-2-TFA, the entire reaction of both compounds is kept in separate channels i.e. the computer monitors 121 independently from each parent separate.

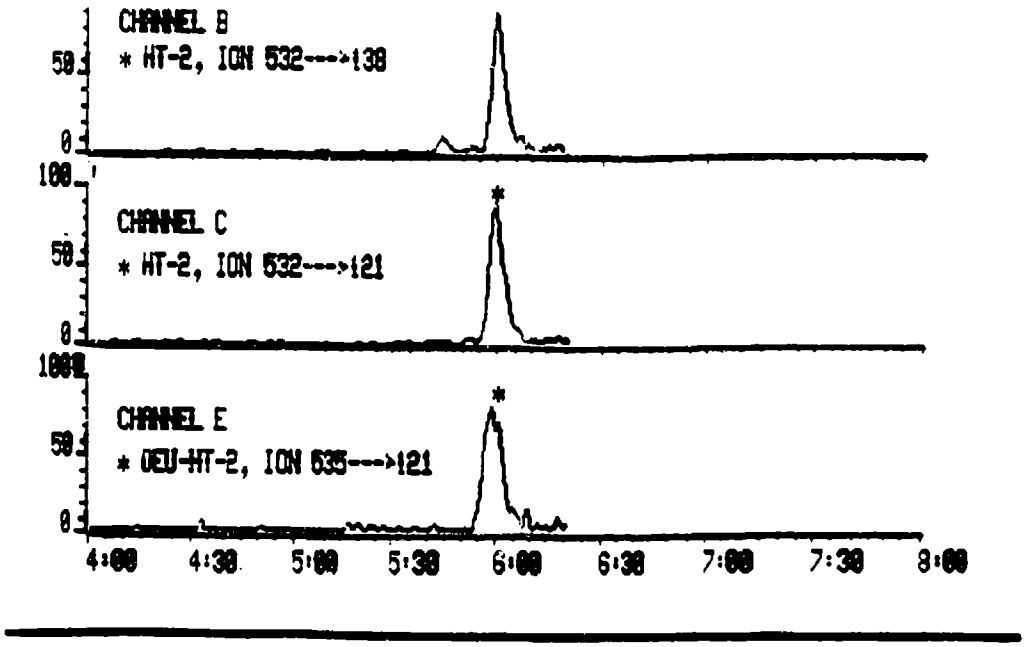
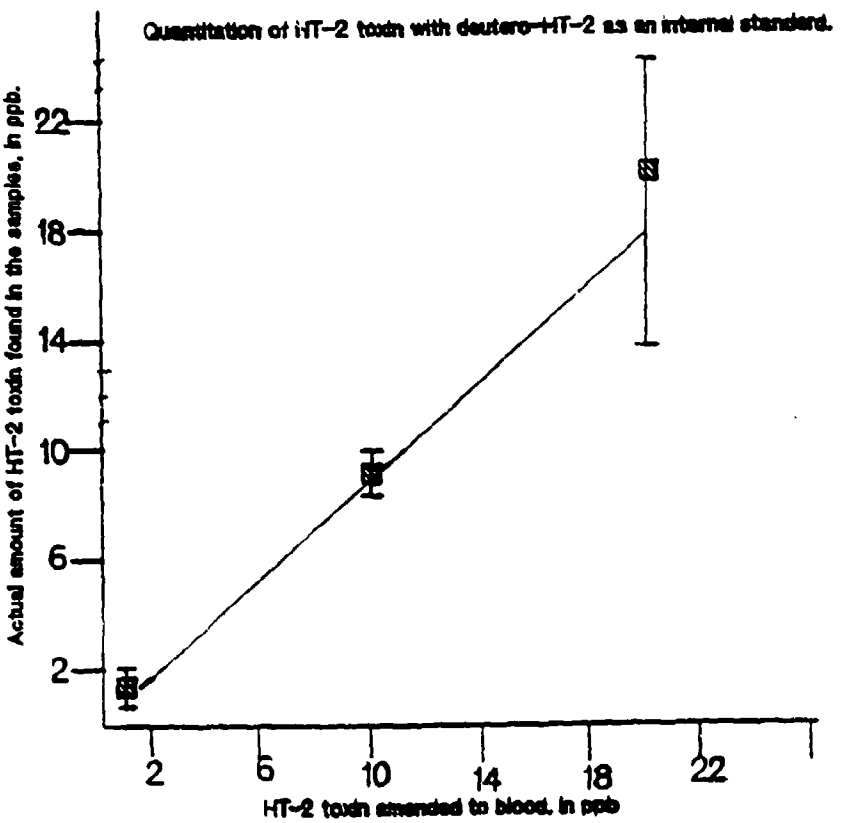


Figure 19. Analysis of human blood amended with HT-2 toxin at 1, 10 and 20 ppb (ng/ml). The analysis was conducted using MRM and the calculations are based on an internal deuterated standard of HT-2 toxin. The percent recovery at 1 and 10 ppb is 70-80% with good accruacy and precision.



SYNTHESIS OF DEUTERATED T-2, HT-2 AND ACETYL-T-2 TOXINS

INTRODUCTION

Analyses of complex biological mixtures such as those represented by blood, urine and animal feeds often generates data that may be qualitatively correct but quantitatively questionable. The coefficient of variation may be up to 100% in terms of quantitation, particularly at levels approaching 50 ppb and less. As an example, the trichothecenes mycotoxins present special problems in analysis because usually they are present as trace components and are detected as trimethyl silyl ether or trifluoroacetate derivatives. There is an inherent error in derivative formation especially when reactions are carried out in a biological matrix. Moreover, the errors are compounded by errors made in the various clean-up and extraction procedures and thus each separate procedure must be analyzed and percent recovery determined. Finally, the percent recovery is generated and a correction factor is calculated. The synthesis of deuterated standards of T-2, HT-2 and acetyl-T-2 is the subject of this report.

MATERIALS AND METHODS

Synthesis of deuterated acetyl-T-2 (3-d-acetoxy-T-2) [3] (Fig. 24). A mixture of T-2 [1] toxin (100 mg), dimethylamino pyridine (30 mg), ethylene dichloride (0.4 ml) and deuterated acetic acid anhydride (0.4 ml) was stirred for 24 hrs at room temperature (22-24 C). The reaction products were filtered through a short Pasteur pipette containing silica gel and eluted with methylene dichloride. The elution solvent was evaporated under vacuum and the crude product was

resolved on a 10 gram silica gel column. It was eluted with ethyl acetate:petroleum ether (2:3) and yielded 84 mg of acetyl-T-2 toxin [3] (76.6% yield). The molecular weight of the product was determined by mass spectrometry as M+ = 511 (508 + 3). The empirical formula of [3] = C26 D3 H33 O10 (Fig. 20).

Synthesis of deuterated diacetyl-T-2[3,4-(deutero)-diacetoxy-T-2] [4]. A mixture of HT-2 [2] (82 mg), dimethylamino pyridine (20 mg), ethylene dichloride (4 ml) and deuterated acetic anhydride (0.5 ml) was stirred for 20 hrs at 21-23 C. The solvent was evaporated under vacuum to give a crude product which was resolved on a chromatographic column (10 grams silica gel). The product was eluted with ehtyl acetate:petroleum ether (2:3) to yield 75.8 mg of [4] with a 75.8% yield. The molecular weight (M+ = 514 [508 + 6] was determined by mass spectrometry. The empirical formula of [4] = C26 D6 H30 O10 (Fig. 21).

Synthesis of deuterated T-2 (4,15-di-deutero-acetoxy) [9] and deuterated HT-2(15-deutero-acetoxy) [10]. A 2% solution of $NaHCO_3$ (0.35 ml) was added to a solution of [8] (21.6 mg) in methanol (4.0 ml) and stirred for 70 min at 21-23 C. The reaction mixture was extracted with ethyl acetate (30 ml), washed with water and dried with anhydrous Na_2SO_4. The solvent was removed to yield a crude product weighing 18.7 mg; it was resolved on a chromatographic column (10 g silica gel) and eluted with varying ratios of ethyl acetate:petroleum ether solvent system. The fractions of effluent were monitored by TLC for products of the reaction. The fractions containing each product were then combined and concentrated under vacuum to yield: [9] (4,15-di-deutero-acetoxy-T-2); 9.3 mg, 47% yield; C_{24} D_6 H_{28} O_9; M+ = 472 +

6; Trifluoroacetate derivative (TFA) of [9] = C26 D6 H27 F3 O10; M+ = 568 (562 + 6). [10] Deuterated HT-2 (5-deutero-acetoxy-HT-2; (7.6 mg and 42.6% yield); C_{22} D_3 H_{29} O_8; M+ = 427 (424 + 3); TFA derivative of [10] = C_{26} D_3 H_{27} O_{10} F_6; M+ = 619 (616 + 3) (Fig. 22).

Synthesis of deuterated T-2 (4-deutero-acetoxy) [5]. A 2% solution of NAHCO3 (0.32 ml) was added to a solution of [4] (39.4 mg) in methanol 98 ml). The mixture was stirred for 90 min at room temperature and then the reaction was stopped by quenching with 10 ml water and 0.5 ml acetic acid. After removal of the methanol, the reaction mixture was extracted with ethyl acetate (50 ml) and washed with 5% aqueous $NaHCO_3$ solution and then dried over anhydrous sodium sulfate. The preparation was taken to dryness in a rotary evaporator and yielded a crude product (36 mg). The latter was chromatographed on a 10 g silica gel column and eluted with ethyl acetate:petroleum ether. The effluent was monitored by TLC and the appropriate fractions were combined and evaporated in vacuo. The yield of [5] was 26.3 mg (73.1%) and the yield of [2] was 4.0 mg (12.3%). Empirical formula of [5]: C_{24} D_3 H_{31} O_9; M+ = 468 (466 + 3). The TFA derivative of [5] yielded [6] with an empirical formula of C_{26} D_3 H_{30} F_3 O_{10}; M+ = 565 (562 + 3).

Synthesis of deuterated-acetyl-T-2(3,4,15-tri-d-acetoxy-T-2) [8]. A mixture of T-2-triol [7] (21 mg) DMAP (6 mg), ethylene dichloride (3 ml) and deuterated acetic anhydride (0.6 ml) was stirred for 20 hrs at room, temperature (21-23 C). After removal of the solvent under vacuum, the reaction mixture was chromatographed on a 5 g silica gel column and eluted with ethyl acetate-petroleum ether. The

yield of [8] was 21.6 mg (77.2%). The empirical formula of [8] = C_{26} D_9 H_{27} O_{10}; M+ = 517 (508 + 9 + 1) (Fig. 23).

The standards described above were used as internal standards in the multiple reaction monitoring of T-2 toxin in blood studies.

The mass spectra of deuterated T-2, acetyl-T-2 and HT-2 are shown in Figures 1 (3-d-acetyl-T-2), Figure 2 (3,15-d-diacetyl-T-2), Figure 3 (15,d,acetyl-HT-2), Figure 4 (3-d-acetyl-T-2) and Figure 5 (structural formulas of the derivatives and final products----).

The nomenclature, empirical formulas and molecular ion information is provided in Table 4.

Table 4. Nomenclature, empirical formul.. ..id molecular ions of various trichothecene derivatives made in the synthesis of the deuterated standards.

Compound	Empirical Formular	MS M^+	(TFA der.) MS M^+
Acetyl-T-2	$C_{26}H_{36}O_{10}$	508	
Deuterated-Acetyl-T-2			
(3-d-acetoxy)	$C_{26}D_3H_{33}O_{10}$	511	
Deuterated-Acetyl-T-2			
(3,4-di-d-acetoxy)	$C_{26}D_6H_{30}O_{10}$	514	
Deuterated-Acetyl-T-2			
(3,4,15-tri-d-acetoxy)	$C_{26}D_9H_{27}O_{10}$	517 + 1	
T-2	$C_{24}H_{34}O_9$	466	562
Deuterated-T-2			
(4-d-acetoxy)	$C_{24}D_3H_{31}O_9$	469	565
Deuterated-T-2			
(4,15-di-d-acetoxy)	$C_{24}D_6H_{28}O_9$	472	568
HT-2	$C_{22}H_{32}O_8$	424	616
Deuterated-HT-2			
(15-d-acetoxy)	$C_{22}D_3H_{29}O_7$	427	619

Figure 20. Trifluoroacetate derivative of 3-d-acetate-T-2 toxin. The TFA group is in the C-3 position and the deuterated (d=3) acetate on C-4. The M+ of TFA-T-2 = 562 and the deuterated standard is 565.

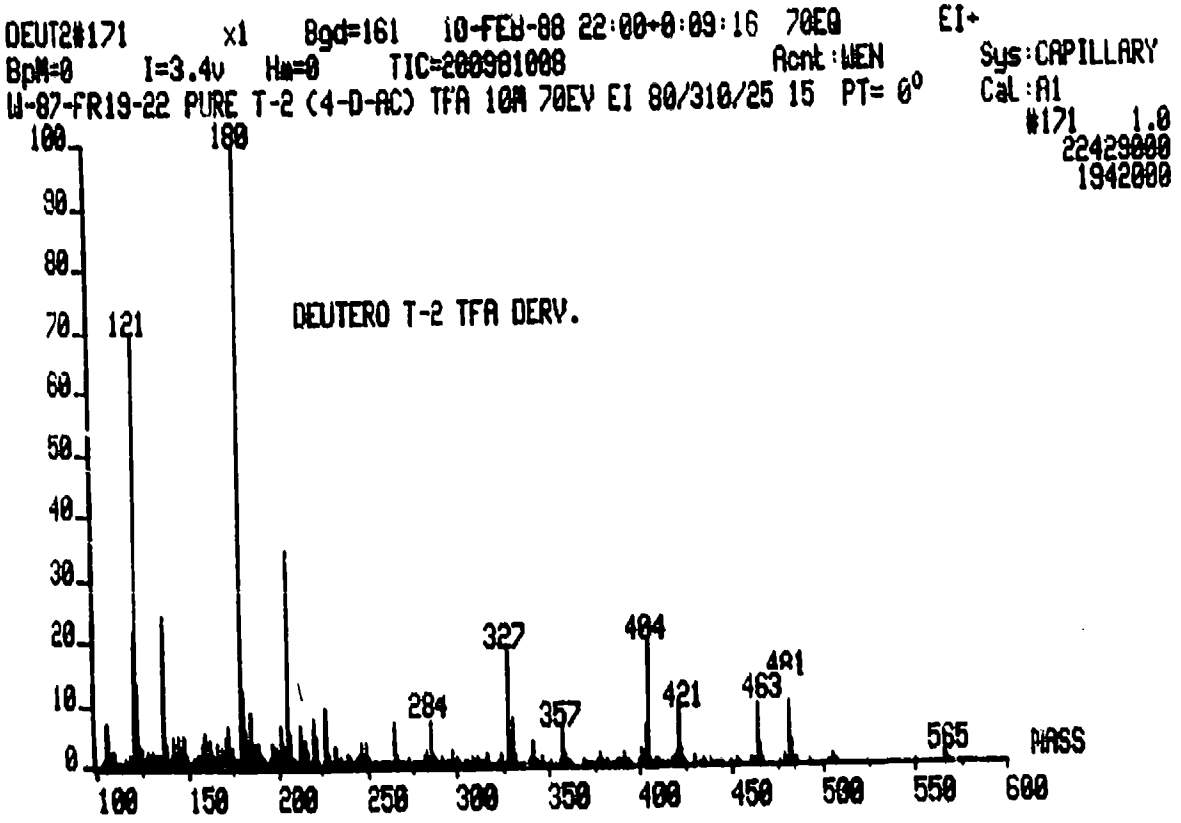


Figure 21. Trifluoroacetate ester of 3,15-d-diacetyl-T-2. The TFA group is on C-3 whereas the deuterated acetate groups are on C-4 and C-15. The M+ of the TFA-T-2 is 568 (562 + 6).

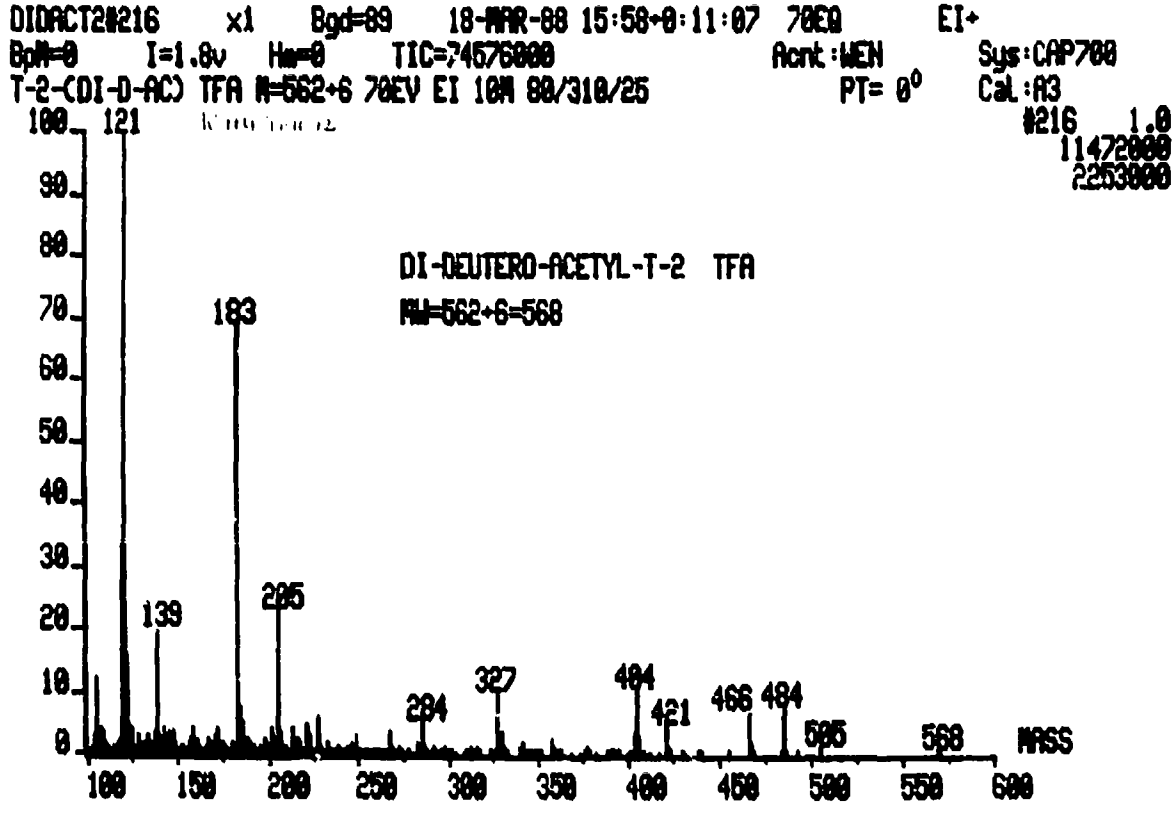


Figure 22. Trifluoroacetate ester of 15-d-acetate-HT-2. The TFA groups are on C-3 and C-4 whereas the deuterated acetate is on C-15. The M+ = 616 + 3 = 619.

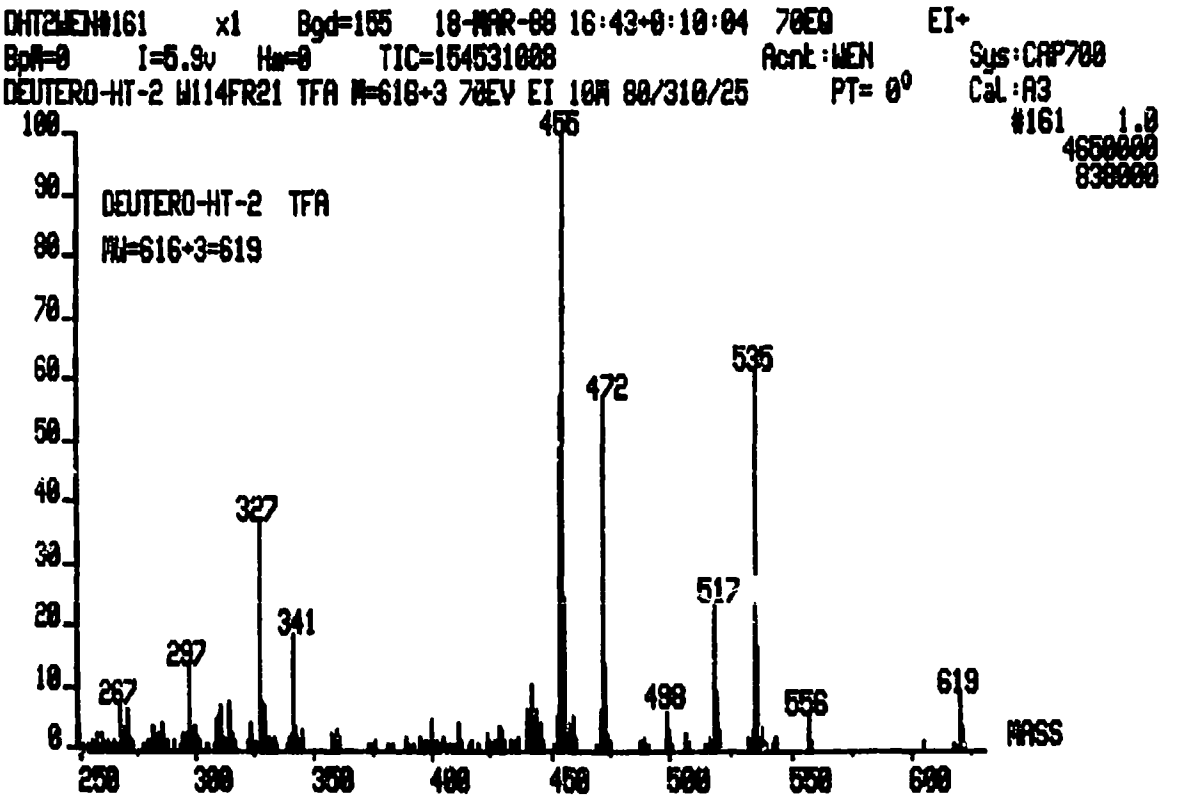


Figure 23. Deuterated acetyl T-2 (3-d-acetoxy-T-2). The deuterated acetate group is on C-3; M+ = 508 + 3 = 511.

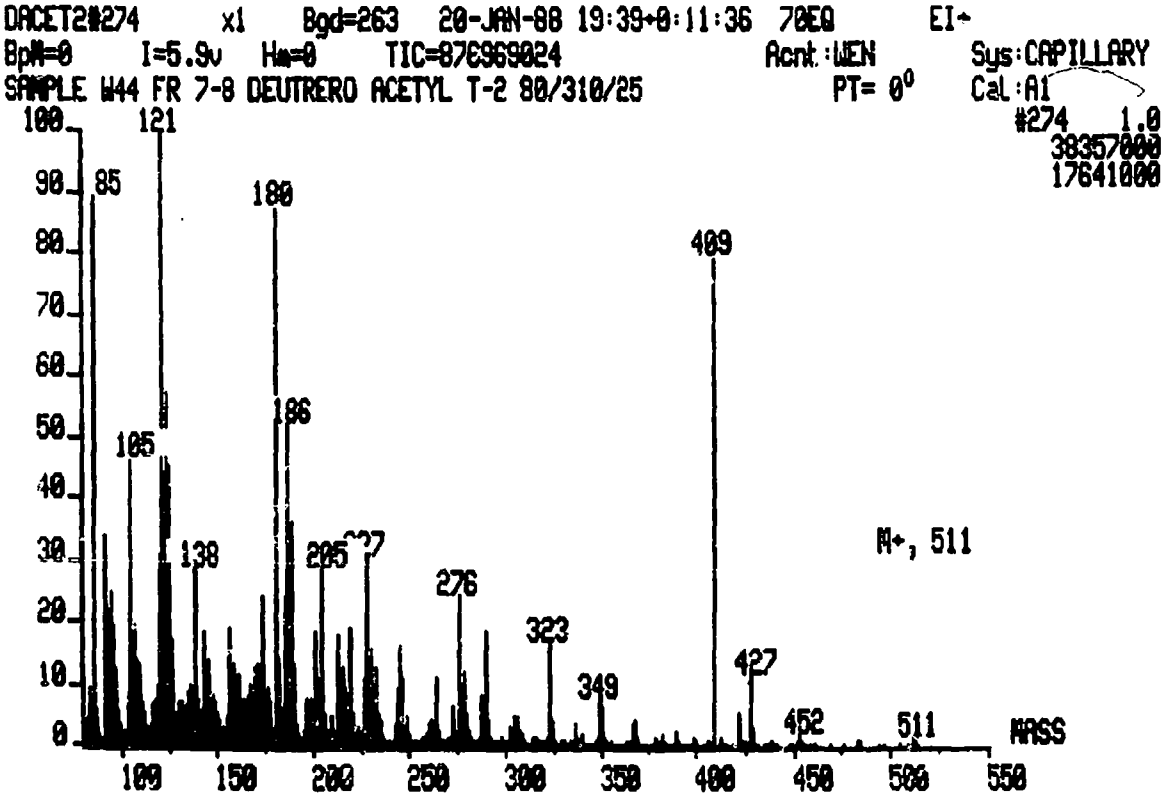
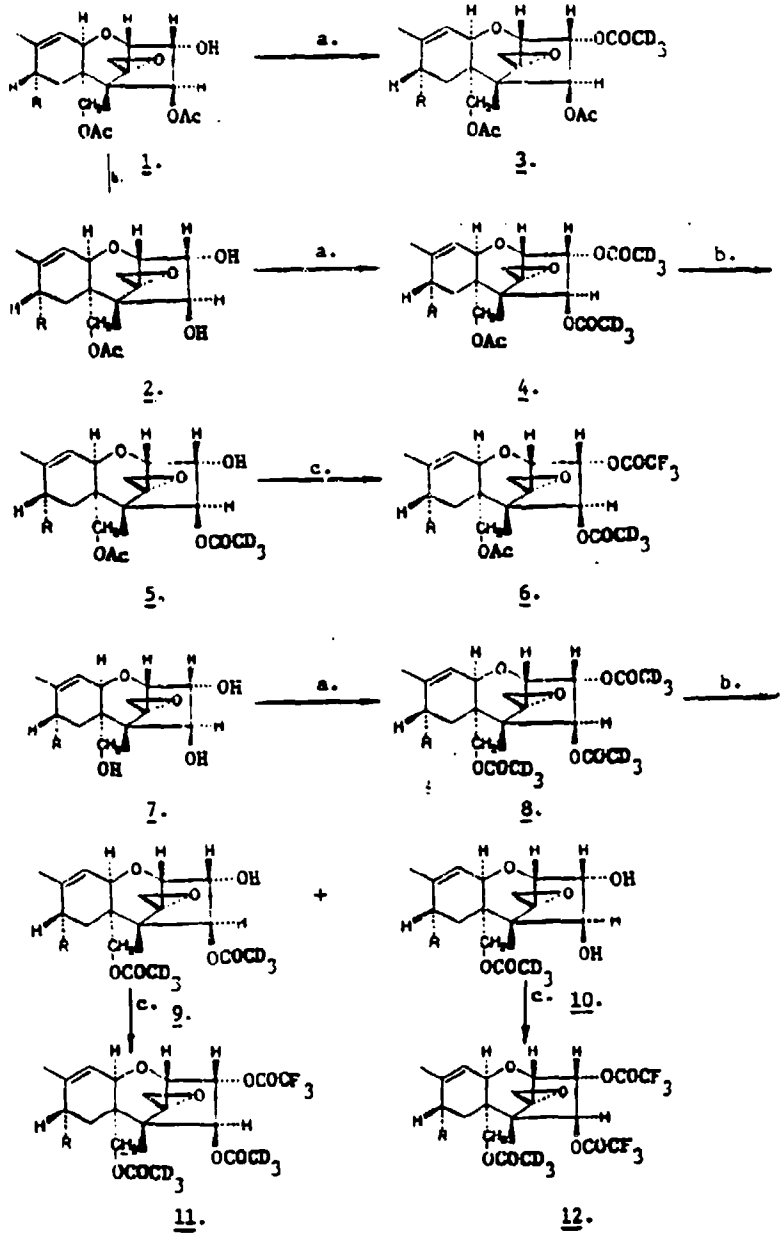


Figure 24. Structural formulas of the derivatives and final products of the synthesis of the deuterated standards.



a. $(CD_3CO)_2O$, CH_2ClCH_2Cl, DMAP b. 2% $NaHCO_3$ SOL. MEOH c. $(CF_3CO)_2O$

PATHOLOGIC EFFECTS OF WORTMANNIN (H-1 TOXIN) PRODUCED BY FUSARIUM OXYSPORUM

A culture of Fusarium oxysporum obtained from the Arctic region of Norway produced hemoglobinuria and death when fed to weanling rats. The toxic principle was found to be a single, novel toxin, which we have designated H-1 toxin. Intragastric gavage of partially purified and purified toxin produced hemorrhage in the gastrointestinal tract, thymus and myocardium. Myocardial hemorrhage was severe and transmural, and may be of diagnostic significance. The major microscopic lesions were hemorrhage and necrosis of lymphocytes in the thymus, spleen and lymph nodes. Necrosis of gastrointestinal epithelium was not seen.

INTRODUCTION

The fungal genus Fusarium produces several toxic metabolites, including trichothecene mycotoxins (Marasas et al. 1984). Fusarium oxysporum is a common contaminant of vegetables, fruit and cereal crops and has been associated with toxicity to several species of animals (Abbas et al. 1984, 1987; Diener et al. 1976; Ghosal et al. 1976). Extracts of this species were found to contain trichothecenes, including diacetoxyscirpenol and T-2 toxin (Ghosal et al. 1976). Tuttobello et al. (1974) also found estrogenic activity in extracts of F. oxysporum, but did not identify the causative component. We observed profound toxicity of 25 of 31 isolates of F. oxysporum isolated from the arctic region of Norway, when fungus-contaminated rice was fed to weanling rats (Abbas et al. 1987). Extraction of the fungal cultures failed to produce any known mycotoxins, but produced a

novel metabolite empirical formula $C_{23}H_{24}O_8$, (mw 428) which proved to be highly toxic to weanling rats (Abbas and Mirocha, 1987). The objective of the present study was to record the spectrum of lesions produced by crude and purified toxin, designated as H-1 toxin, in rats. The major lesions we produced in rats were hemorrhage, hemoglobinuria and necrosis of lymphocytes. Severe hemorrhage of the myocardium was a commonly found lesion.

MATERIALS AND METHODS

Animals. Twenty day, female Sprague-Dawley rats (Rattus norvegicus, Bio-lab, White Bear Lake, MN) were used for all experiments.

Feeding tests. These were performed with a mixture of complete feed and rice medium on which F. oxysporum had been grown for two weeks as described by Abbas et al. (1984). This rice medium contained 43 ppm (wt/wt) H-1 toxin, as determined by HPLC. A single isolate (designated N17B), which was previously shown to be highly toxic to rats and did not produce any other known Fusarium toxins, was used for all feeding trials (Abbas et al. 1987). Three to one, 1:1 and 1:3 mixtures of finely ground toxic rice cultures and complete rat diet were fed ad libitum for five days to three groups of 8 rats; controls were fed a 1:1 mixture of culture-medium and complete diet (control diet). Thirty-one additional rats were fed the 1:1 ration for four days in order to collect urine (Table 5). Urine from 24 rats which produced dark-red urine following the four day feeding trial with toxic feed was pooled into three lots and tested for the presence of hemoglobin by $(NH_4)_2SO_4$ precipitation (Blondheim et al. 1958). Blood

smears from 8 rats on this same diet and 6 control rats were stained with Diff-Quik (Dade Diagnostics, Aguada, PR) formicroscopic evaluation and the packed cell volume of the blood was determined by the microhematocrit method (Raphael, 1976). All rats were necropsied shortly after death and portions of brain, thymus, heart, lung, liver, spleen, ovary, kidney, urinary bladder, adrenal, pancreas, stomach, duodenum, jejunum, ileum, colon, mesenteric lymph node and bone marrow of several rats per group were fixed in 10% neutral, buffered formalin, embedded in paraffin and processed routinely for microscopic evaluation.

Gavage of toxic fractions. Extraction and purification was done as described by Abbas and Mirocha (1987). Briefly, crude toxic or control extract was prepared by filtration of an ethyl acetate extract of the contaminated rice medium described above or of control medium through Whatman R number 4 filter paper, followed by extraction with water, dissolution in acetonitrile and delipidation with petroleum ether. The acetonitrile fraction was dried and dissolved in 40% ethanol for intragastric gavage. Further purification of the toxic fraction was by chromatography on a florisil column by the method of Tanaka et al. (1985), followed by thin layer chromatography in chloroform-methanol (97:3) on silica gel plates (C.E. Merck, Darmstadt, Fed. Rep. Ger.). TLC bands were scraped off the plates and eluted with absolute ethanol. Dried florisil fractions and TLC fractions were dissolved in 40% ethanol for intragastric gavage. Dried TLC eluates were recrystallized 3 times from methanol to give a preparation which was more than 95% pure as assayed by high pressure

liquid chromatography (unpublished information) and dissolved in 40% ethanol for intragastric gavage (purified toxin). A single dose (equivalent to the extract of 2.5 g of rice-medium culture) of crude toxic or control extract, each of 4 florisil fractions and each of 10 TLC fractions was given in 500 microliters of 40% ethanol to 9, 6 and 6 rats, respectively. Purified toxin was given in single doses of 25 mg/kg body weight (2 rats), 50 mg/kg (2 rats) and 4 mg/kg (12 rats, Table 5). Gavage-controls received 500 microliters of 40% ethanol and 2 groups of six rats were fed the residues of ethyl acetate-extracted toxic feed or control feed. Animals were necropsied 24 hours post-gavage or after five days feeding of the residues. Tissues were processed for microscopic evaluation as before.

RESULTS

Feeding tests. Most animals died or were killed when moribund (Table 6). The most consistent gross lesion, found in 37 of 55 animals, was distension of the urinary bladder by very dark urine, whose pigment was identified as hemoglobin. One animal showed acute hemorrhage of the stomach and two other animals showed acute hemorrhage of the small intestine (Table 7). Significant microscopic lesions were limited to lymphoid tissue, with 6 of 6 rats showing moderate necrosis of germinal centers in the spleen, lymph nodes and gut-associated lymphoid tissue, as well as depletion and mild necrosis of thymic lymphoid tissue. Two rats showed mild, acute necrosis of proximal renal tubular epithelium (Table 8). There were no abnormalities on the blood smears and differential blood counts, and packed erythrocyte volumes were within normal limits. No deaths or

lesions occurred in the controls.

Gavage. The crude extract, partially purified and purified toxin were highly toxic (Table 6). Toxicity of the florisil preparation was limited to one of four 75 ml fractions and thin layer chromatography of this fraction produced 10 bands, of which one was toxic. Recrystallization of this band produced fluffy, white purified toxin. All toxic fractions produced bright yellow solutions in 40% ethanol.

Gross lesions were limited to severe hemorrhage of several tissues, with severe myocardial and gastric hemorrhage the most common lesions (Table 7, Figure 25). Only one animal had hemoglobinuria. Major microscopic lesions included severe hemorrhage in the myocardium and submucosa of the stomach, and necrosis of lymphoid tissue (Table 8). The txtent of myocardial hemorrhage ranged from focal subendocardial involvement to, more commonly, locally extensive to diffuse involvement of both ventricles and atria. Lymphoid necrosis was severe in the cortex of the thymus and was often accompanied by locally extensive hemorrhage. There was moderate necrosis of lymphocytes in the cortex and paracortex of lymph nodes, in the periphery of Peyer's patches of the ileum and in germinal centers in the spleen. The periarteriolar lymphoid sheaths of the spleen were relatively spared. Occasional necrotic epithelial cells were present in the zona fasciculata of the adrenal cortex in a few animals. Kidney lesions consisted of a few foci of necrosis of proximal tubular epithelium. No deaths or lesions occurred in the controls.

DISCUSSION

The spectrum of lesions produced by H-1 toxin of Fusarium oxysporum is, in many respects, similar to those produced by other Fusarium mycotoxins, but also has significant differences. Other Fusarium mycotoxins, notably the trichothecenes, will also produce lymphoid necrosis and have been reported to cause acute hemorrhage (Brenrecke et al. 1982, Friend et al. 1983, Mirocha, 1984, Petrie et al. 1977, Sato et al. 1975, Schiefer et al. 1985, Weaver et al. 1981). However, these radiomimetic toxins also produce necrosis of gastrointestinal epithelium and bone marrow, which were not found in our cases. The severe myocardial hemorrhage seen in our rats is an unusual lesion, similar to that produced by vitamin E/selenium deficiency in pigs, which may be of diagnostic importance in distinguishing H-1 toxicosis from trichothecene toxicosis (Jubb and Kennedy, 1971). Natural and experimental exposure to trichothecenes has caused hemorrhage diarrhea, mucosal and subcutaneous hemorrhage and hemorrhage in the epicardium, but deep myocardial hemorrhage has not been a feature of toxicosis due to this class of mycotoxins (Kosuri et al. 1971; Pier et al. 1976). In our experience, administration of trichothecenes has also not caused this type of lesion in rats or other animals (unpublished information). In screening tests of fungal isolates from various areas of the world, we have found several instances of severe myocardial hemorrhage in rats fed medium on which these isolates had been cultured and in all these cases, H-1 toxin was subsequently found in the media (unpublished information). The variability in the incidence of myocardial

hemorrhage in our studies may be related to differences in dose, administration schedule or susceptibility of individual animals.

We found no obvious cause of the hemorrhage. There was no light-microscopic evidence of vascular damage and the number of platelets on blood smears of the rats on the feeding trials, as well as the number of megakaryocytes in bone marrow of rats of all groups, were within normal limits. Decreased activities of clotting factors and dysfunction of platelets have been found in mycotoxicosis due to trichothecenes, but we did not investigate these possibilities (Chan and Gentry, 1984; Gentry and Cooper, 1982; Yarom et al. 1984). We also did not determine the cause of the hemoglobinuria which was seen in many rats in the feeding tirals. Since H-1 toxin caused hemorrhage in the gavaged rats, occult hemorrage is the most likely cause of the hemoglobinuria and the higher incidence of this condition in the rats of the feeding trials may have occurred because the animals on the other trials died too quickly for this lesion to develop. We have seen hemoglobinuria in cats with long-term administration of low levels of T-2 toxin and have also observed it in acute trichothecene toxicity in rats, indicating that this particular condition is not unique to H-1 toxin (unpublished information). In those cases, hemoglobinuria was associated with frank gastrointestinal bleeding, but we saw no evidence of such in our feeding trials.

The hemorrhagic lesions produced by H-1 toxin suggest that this compound should be considered as a cause of unexplained hemorrhage in livestock. Unexplained hemorrhage, especially gastrointestinal, of farm animals is fairly commonly reported (Blood et al. 1983).

Mycotoxins have been postulated to be the causative agents in some of these instances, but no solid data has been produced to support this contention (Petrie et al. 1977; Weaver et al. 1980). In addition to its hemorrhagenic effect, H-1 toxin severely affects lymphoid tissue. This deleterious affect suggests that H-1 toxin may decrease resistance to disease and is thus a potential factor in the prevalence and severity of human and animal diseases. Preliminary screening tests in our laboratory have demonstrated the presence of H-1 toxin in cultures of fungi from regions other than the arctic area of Norway, suggesting that this toxin may be widely distributed and may therefore be potentially important as a food-borne hazard to the health of humans and animals.

REFERENCES

Abbas HK, Mirocha CJ, Berdal BP, Sundheim L, Gunther R, and Johnson B (1987) Isolation and toxicity of Fusarium species from various areas of Norway. Acta Agric. Scand. 37:427-435.

Abbas HK, and Mirocha CJ (1987) Isolation and purification of a hemorrhagic factor (H-1) from Fusarium oxysporum (N17B). Appl. Environ. Microbiol. 54:1268-1274.

Abbas HK, Mirocha CJ, and Shier WT (1984) Mycotoxins produced from fungi isolated from foodstuffs and soil: Comparison of toxicity in fibroblasts and rat feeding tests. Appl. Environ. Microbiol. 48:654.

Blondheim SH, Margoliash E, and Shafrir E (1958) A simple test for myohemoglobinuria (myoglobinuria). J. Am. Med. Assoc. 167:453.

Blood DC, Radostits OM, and Henderson JA (1983) Veterinary Medicine, 6th Ed., p. 136. Philadelphia: Bailliere Tindall.

Brenrecke LH, and Neufeld HA (1982) Pathologic effects and LD_{50} doses of T-2 toxin in rats by intramuscular, subcutaneous and intraperitoneal routes of administration. Fed. Proc. 41 (Abstracts), 920.

Chan PK, and Gentry PA (1984) Inhibition of bovine platelet function by T-2 toxin, HT-2 toxin, diacetoxyscirpenol and deoxynivalenol. Fd. Chem. Toxic. 22:643.

Diener UL, Wagener RE, Morgan-Jones G, and Davis ND (1976) Toxigenic fungi from cotton. Phytopath. 66:514.

Friend SC, Hancock DS, Schiefer HB, and Babiuk LA (1983) Experimental T-2 toxicosis in sheep. Can. J. Comp. Med. 47:291.

Gentry PA, and Cooper ML (1982) Effect of intravenous administration of T-2 toxin on blood coagulation in calves. Am. J. Vet. Res. 44:741.

Ghosal S, Chakrabarti DK, and Basu-Chaudhary KC (1976) Toxic substances produced by Fusarium I. J. Pharm. Sci. 65:160.

Jubb KVF, and Kennedy PC (1971) Pathology of Domestic Animals, 2nd Ed., p. 117. New York: Academic Press.

Kosuri NR, Grove MD, Yates SG, Tallent WR, Ellis JJ, Wolf IA, and Nichols RE (1970) Response of cattle to mycotoxins of Fusarium tricinctum isolated from corn and fescue. J. Am. Vet. Med. Ass. 157:938.

Kurtz HJ (1981) Scirpenols (Trichothecenes). In: Current Veterinary Therapy, Food Animal Practice, 1st ed., p. 410 (Howard E, Ed.), Philadelphia, W.B. Saunders.

Marasas WFO, Nelson PE, and Tousson TA (1984) Toxigenic Fusarium Species. University Park, Pennsylvania State University Press.

Mirocha CJ (1984) Mycotoxicosis associated with Fusarium sp. In: The Applied Mycology of Fusarium, p. 141 (Moss MD, and Smith JE, Ed.), Cambridge University, Cambridge Press.

Mirocha CJ, Pathre SV, Pawlosky RJ and Hewetson DW (1986a) Mass Spectra of Selected Trichothecenes. In: Cole RJ (ed) Modern Methods in the Analysis and Structural Elucidation of Mycotoxins. Academic Press, Inc., New York, 13:354-392.

Mirocha CJ, Pawlosky RJ and Hewetson DW (1986b) Gas Chromatography Mass Spectral Analysis of Trichothecenes. In: Richard JL and Thurston JR (eds) Diagnosis of Mycotoxicoses. Martinus Nijhoff Publ. Boston, 26:305-322.

Petrie L, Robb J, and Stewart AF (1977) The identification of T-2 toxin and its association with a hemorrhagic syndrome in cattle. Vet. Rec. 101:326.

Pier AC, Cysewski SJ, Richard JL, Baetz AL, and Mitchell L (1976) Experimental mycotoxicosis in calves with aflatoxin, ochratoxin, rubratoxin and T-2 toxin. Proc. U.S. Anim, Hlth. Ass. 80:130.

Plattner, RD (1986) Mass Spectrometry-Mass Spectrometry as a Tool for Mycotoxin Analysis. In: Cole RJ (ed) Modern Methods in the Analysis and Structural Elucidation of Mycotoxins. Academic Press, Inc., New York, 14:393-413.

Raphael SS (1976) Lynch's Medical Laboratory Technology, 3rd Ed., p. 1111. Philadelphia: W.B. Saunders.

Sato N, Ueno Y, and Enomoto M (1975) Toxicological approaches to the toxic metabolites of Fusaria, VIII. Acute and subacute toxicities of T-2 toxin in cats. Jpn. J. Pharmacol. 25:263.

Schiefer HB, Nicholson S, Kasali OB, Hancock DS, and Greenhalgh R (1985) Pathology of acute 3-acetyldeoxynivalenol toxicity in mice. Can. J. Comp. Med. 49:315.

Tanaka T, Hasegawa A, Matsuki Y, Ishii K, and Ueno Y (1985) Improved methodology for the simultaneous detection of the trichothecene mycotoxins deoxynivalenol and nivalenol in cereals. J. Food Hyg. Soc. Jpn. 26:519.

Tuttobello L, Zavattiero CO, and Macri A (1974) Ricerca di sostanze ad attivita uterotropa con colture diverse di fusarium. Atti. Soc. Ital. Sci. Vet. 28:624.

Vesonder, RF and Rohwedder, WK (1986) Gas Chromatographic-Mass Spectrometric Analysis of Mycotoxins. In: Cole RJ (ed) Modern Methods in the Analysis and Structural Elucidation of Mycotoxins. Academic Press, Inc., New York, 12:335-349.

Weaver GA, Kurtz HJ, Bates FY, Mirocha CJ, Behrens JC, and Hagler WM (1981) Diacetoxyscirpenol toxicity in pigs. Res. in Vet. Sci. 31:131.

Weaver GA, Hurtz HJ, Mirocha CJ, Bates FY, Behrens JC, Robison TS, and Swanson SP (1980) The failure of purified T-2 mycotoxin to produce hemorrhaging in dairy cattle. Can. Vet. J. 21:210.

Yarom R, More R, Eldor A, and Yagen B (1984) The effect of T-2 toxin on human platelets. Toxicol. Appl. Pharmacol. 73:210.

Table 5. Toxin administration schedule

Trial	Toxic preparation	n
1	Feed	55
2	Crude extract	9
3	Florisil fractions(4)	24 (6 per fraction)
4	TLC bands(10)	60 (6 per fraction)
5	Purified toxin	16

Table 6. Deaths produced by toxic fractions

Fraction	Deaths*
Feed	42/55
Crude extract	9/9
Florisil	6/6
TLC	6/6
Pure toxin	12/16

*Expressed as the number of deaths/number of animals on the study.

Table 7. Gross lesions

Fraction	Lesion (hemorrhage)*			
	Hemoglobinuria	Stomach	Thymus	Heart
Toxic feed	37/55	1/55	0/55	0/55
Crude extract	4/9	9/9	3/9	8/9
Florisil	0/6	5/6	3/6	6/6
TLC	0/7	6/7	0/7	6/7
Pure toxin	1/12	6/12	4/12	2/12

*Expressed as the number of animals with the lesion/number examined.

Table 8. Histologic lesions

Fraction	Lesion*				
	Heart	Kidney	Lymphoid tissue	Adr	Stom
Toxic feed	0/6	2/6	6/6	0/6	0/6
Crude ext.	2/4	0/4	4/4	2/4	3/4
Florisil	3/4	3/3	1/4	0/4	1/4
TLC	7/7	0/7	7/7	3/7	4/7
Pure toxin	5/10	1/10	9/10	0/10	2/10

*Expressed as the number of animals with the lesion/number examined

Adr = adrenal

Stom = stomach

Figure 25. Gross lesion found in the rat fed a ration containing H-1 toxin or when intubated with pure toxin. The unique lesion is hemorrhaging and deep seated necrosis in the heart. Hemorrhaging is also seen in the thymus, stomach and bladder.

TOXINS PRODUCED BY FUSARIUM SPECIES IMPLICATED IN ALIMENTARY TOXIC ALEUKIA (ATA)

The classical description of a mycotoxicosis caused by species of Fusarium was described by scientists working in the Orenburg district of the USSR in the 1940's and headed by Professor Abraham Joffe. The problem was called alimentary toxic aleukia which affected (according to the chronicles) thousands of people eating overwintered grain in the Orenburg district of the USSR. The disease problem was narrowed to the fungus Fusarium and subsequent analyses implicated a metabolite called sporofusarogenin. Professor L. E. Olifson of the Chemistry Department of the University of Orenburg used this problem as a portion of his PhD dissertation (CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF POISONOUS MATERIALS OF CEREALS AFFECTED BY THE FUNGUS FUSARIUM SPOROTRICHIELLA- Moscow- 1965) and described the above toxin as steroid. A sample of a crude toxic preparation of sporofusarin was mailed to us (20 March, 1972) by Professor Bilai of the Microbiology Institute in Kiev. We analyzed it and found that the toxic component was T-2 toxin along with T-2-tetraol and zearalenone (Mirocha, C.J. & Pathre, S. V. 1973, Appl. Microbiol. 26:719). Thus, we reported to the scientific community the results of our findings with the conclusion that the toxin attributed by Soviet scientists as causing alimentary toxic aleukia and called sporofusarin was indeed T-2 toxin. We did not find any steroid molecules but indeed after finding T-2, we did not pursue this thesis any further.

The toxicity described by Professor Joffee as being due to Fusarium, presumably sporofusarogenin, was lethal causing hemorrhaging

and death. The authors described petechial hemorrhaging (hemorrhaging immediately under the skin) in test subjects. In 1987, my laboratory described the isolation and identification of a metabolite produced by Fusarium sambucinum and F. oxysporum that was lethal to rats and produced severe hemorrhaging in the heart, stomach, intestines and bladder. The molecule was identified as wortmannin and proved to be a steroid similar in description to that described by Professor Olifson many years ago. I am once again interested in steroid molecules produced by Fusarium, especially wortmannin, as to the possibility that indeed this substance may be identical to that called sporofusarin. The structure of sporofusarin (SPF) and wortmannin (WRTM) are shown for purposes of comparison (Figure 26). Note that SPF has an aldehyde group between ring A and B whereas WRTM has na ether in the same position. The aldehyde could be easily misconstrued as an ether as we had that same problem in arriving with the structure of wortmannin. Moreover, WRTM has a lactone ring and so does SPF but in a different position. It is possible that in the early 1960's when this work was done, that an erroneous assignment could have been given to the lactone function. We are testing the hypothesis that WRTM found in our laboratory could indeed have been given to the lactone function. We are testing the hypothesis that WRTM found in our laboratory could indeed have been the same metabolite as described by Olifson in 1965. If my hypothesis is correct, then Professor Olifson deserves an apology and due credit for his work on this natural product when structure determination was most difficult.

Recently we have tested the hypothesis that scientists working

in the USSR with toxicity of Fusarium may not have been working with pure cultures i.e. they did not single spore the isolates as is still their custom. We inoculated a culture of rice as is our custom with a high producer (Fusarium poae [5098]) of T-2 toxin and one that is a high producer of wortmannin (both F. sambucinum N90A and N94B were tested) i.e. the culture was seeded simultaneously with both isolates. The culture was grown for one month in the conventional manner and then analyzed for both T-2 and wortmannin. Wortmannin was easily found by TLC but T-2 was not readily detected by TLC although it was present in trace amounts. However, HT-2 was readily found by TLC Table 9. This experiment suggests that indeed a mixed culture of Fusarium could produce both T-2 and HT-2 but in small amounts with major synthesis of wortmannin. Cultures such as these would be extremely toxic but the toxicity would be due to wortmannin and not T-2.

A crucial experiment that remains to be done is to determine whether wortmannin could cause pronounced leukopenia as reported in cases of alimentary toxic aleukia (ATA). If it does, then the hypothesis may gain additional credence as to causality of ATA.

Table 9. Determination of trichothecenes and wortmannin from Fusarium cultures grown on rice.

	Mycotoxin concentrations (ppm)				
Mycotoxin	5098	N90A	N94A	5098 + 90A	5098 + 94A
T-2	500	0	0	<1	<1
HT-2	5	0	0	20	25
Wortmannin	0	30	45	25	42
T-2 tetraol	20	0	0	40	45

These results were quantitated by TLC plates. 5098 = F. poae; N90A = F. sambucinum; N94A = F. sambucinum; 5098 + 90A = F. poae + F. sambucinum; 5098 + 94A = F. poae + F. sambucinum.

Figure 26. Comparison of structures of wortmannin (H-1) with sporofusarin and its derivatives

	R1	R2
Lipotoxol	CH3	OH
Sporofusarin	CH3	glucose-glucose-ribose
Poaefusarin	HC=O	xylose